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NON-PROVISIONAL APPLICATION FOR LETTERS PATENT

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**PLANT-DERIVED MOLECULES AND GENETIC
SEQUENCES ENCODING SAME AND USES THEREFOR**

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lla:2/8/02

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February 8, 2002
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EL 571469674 US
Express Mail Tracking Number

**PLANT-DERIVED MOLECULES AND GENETIC SEQUENCES
ENCODING SAME AND USES THEREFOR**

5

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims priority from United States Provisional Application No. 60/267,271, filed February 8, 2001.

FIELD OF INVENTION

10 The present invention provides genetic molecules encoding plant floral defensin-like molecules and their use in generating transgenic plants having resistance or at least reduced sensitivity to plant pests including insects, microorganisms, fungi and/or viruses. The present invention further provides for the use of floral-and seed-derived defensins in the generation of insect resistance in plants. The plants may be monocotyledonous or dicotyledonous plants and are in particular, crop plants and ornamental flowering plants. The genetic molecules are also useful in generating recombinant defensin-like molecules for use in the topical application of compositions to prevent or otherwise retard pest-infestation of plants. The floral defensin-like molecules or genetic molecules encoding same of the present invention may be used alone or in combination with other agents such as a proteinase inhibitor
15 precursor or a nucleic acid molecule encoding same or other molecules or their encoding nucleotide sequences.
20

BACKGROUND OF THE INVENTION

References to any prior art in this specification is not, and should not be taken, as an acknowledgement or any form of suggestion that this prior art forms part of the common
25 general knowledge in Australia or any other country.

The increasing sophistication of recombinant DNA techniques is greatly facilitating research and development in the agricultural industry. This is particularly the case in the horticultural area including the area of crop research. Of particular importance are the development of herbicide resistant plants and the development of pathogen resistant plants.

30 A number of approaches have been adopted to induce herbicide resistance in plants. For example, genes encoding enzymes, which deactivate or neutralize the active components

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of herbicides, have been expressed in plants. Whilst there has been some success in this approach, it is one component of a multi-disciplined and multi-strategy approach to maximizing yields of crops and products of crops and for maximizing returns from other activities within the agricultural and horticultural industries.

5

One of the major difficulties facing the agricultural and horticultural industries is the control of insect and other pathogen infestation of plants. Insects and other pathogens account for millions of tonnes of lost production on an annual basis. Although insecticides and other anti-pathogenic chemical agents have been successfully employed, there is a range of environmental and regulatory concerns with the continued use of chemical agents to control plant pests. Furthermore, the increasing use of chemical pesticides is providing selective pressure for the emergence of resistance in populations of pests. There is clearly a need to further investigate alternative mechanisms of inducing resistance in plants to pathogens such as insects, microorganisms, fungi, arachnid and viruses.

15

A range of genetic measures has been adopted in test trials. Whilst some success has been achieved, it is important for new and alternative genetic approaches to be developed to combat the difficulties of resistance.

20

One approach which has been suggested is the use of a group of proteins collectively known as "defensins". The defensins have previously been known as γ -thionins and are structurally distinct from the α - and β -thionin families. Most defensins isolated and studied to date have been derived from seeds, especially those from *Raphanus sativus* and other members of the Brassicaceae family. Seed defensins are small (~5 kDa) basic, cysteine-rich proteins and many have anti-fungal activity.

25

Over the last few years several cDNA clones have been isolated from the floral organs of solanaceous plants and *Arabidopsis* that encode proteins that are related to seed defensins. Unlike seed defensins, floral defensins are produced from precursor proteins that have an acidic C-terminal domain in addition to the defensin domain. The role of this acidic domain is unknown.

30

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- The defensin domain has little sequence in common with seed-derived defensins apart from eight cysteine residues that are strongly conserved. Although several cDNAs, which encode floral defensins, have been isolated, the corresponding proteins have not been isolated and their biological function has not been examined. The inventors have isolated both the cDNA and the corresponding floral defensin from the ornamental tobacco *Nicotiana glauca*. They have determined that the defensin precursor is processed proteolytically to release mature defensin from the acidic C-terminal domain.
- 10 In accordance with the present invention, the inventors have determined that floral defensins have useful properties in inhibiting plant pest attack or infestation. Furthermore, a floral defensin from *Nicotiana glauca* is shown to be particularly effective in controlling insect attack. The present invention also provides a new use of seed and known floral defensins in the control of insect infestation of plants.

SUMMARY OF THE INVENTION

Throughout this specification, unless the context requires otherwise, the word comprise, or variations such as "comprises" or "comprising", will be understood to imply the inclusion
5 of a stated element or integer or group of elements or integers but not the exclusion of any other element or integer or group of elements or integers.

Nucleotide and amino acid sequences are referred to by a sequence identifier number (SEQ ID NO:). The SEQ ID NOs: correspond numerically to the sequence identifiers <400>1,
10 <400>2, etc. A sequence listing is provided after the claims.

The present invention relates generally to genetic molecules alone or in combination with other genetic molecules and their use to induce resistance in plants or parts of plants to pathogen infestation such as but not limited to insect infestation. More particularly, the
15 present invention provides genetic molecules encoding defensin-like molecules alone or in combination with genetic molecules encoding a proteinase inhibitor or precursor thereof or other active molecule to inhibit insect, microbial, fungal, arachnid or viral attack or other form of infestation in plants. The present invention further encompasses compositions comprising the defensin-like molecules alone or in combination with a proteinase inhibitor
20 or precursor thereof or other active molecule for topical application to plants or parts of plants to assist in the control of insect, microbial, fungal, arachnid or viral infestation of plants. The present invention further contemplates the use of the subject genetic molecules in the manufacture of transgenic plants with resistance or at least reduced susceptibility to insect, microbial, fungal, arachnid or viral attack or other form of infestation. The defensin
25 molecules may also be used as molecular frameworks to carry heterologous amino acid sequences where the folding of the molecule is altered to a more active form. The present invention further encompasses genetic constructs comprising a promoter and/or other regulatory sequence naturally associated with the gene encoding the defensin-like molecule. The promoter and/or other regulatory sequence may be operably linked to a
30 cDNA molecule encoding the defensin-like protein or may be operably linked to another gene or nucleotide sequence of interest such as but not limited to a gene encoding a

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proteinase inhibitor precursor. The present invention still further extends to transgenic plants or parts of transgenic plants with resistance or at least reduced sensitivity to attack or other form of infestation by insects, microorganisms, fungi and/or viruses. Particularly preferred plants are food and non-food crops such as cotton plants.

5

Accordingly, one aspect of the present invention provides an isolated nucleic acid molecule comprising a sequence of nucleotides encoding or complementary to a sequence encoding a polypeptide comprising, in its precursor form, an N-terminal signal domain, a mature domain and an acidic C-terminal domain wherein said polypeptide is produced during flower development and its mature domain has activity against one or more plant pests.

10

Another aspect of the present invention is directed to an isolated nucleic acid molecule comprising a sequence of nucleotides encoding or complementary to a sequence encoding a polypeptide comprising, in its precursor form, an N-terminal signal domain, a mature domain and an acidic C-terminal domain wherein said polypeptide is produced during flower development and its mature domain comprises the structure:-

15

$$a_1a_2C_Ia_3a_4a_5a_6a_7a_8a_9a_{10}a_{11}a_{12}C_{II}a_{13}a_{14}a_{15}a_{16}a_{17}C_{III}a_{18}a_{19}a_{20}C_{IV}a_{21}a_{22}a_{23}a_{24}a_{25}$$
$$a_{26}a_{27}a_{28}a_{29}C_Va_{30}a_{31}a_{32}a_{33}a_{34}a_{35}C_{VI}a_{36}C_{VII}a_{37}a_{38}a_{39}C_{VIII}$$

20

wherein "a" may be the same or different and represents any amino acid residue, the numerical subscript on each "a" represents its position in the amino acid sequence and "C" represents a cysteine residue at a position indicated by its Roman numeral and wherein the mature domain has activity against one or more plant pests with the proviso that the polypeptide is not FST or TAP3.

25

A further aspect of the present invention contemplates an isolated nucleic acid molecule comprising a sequence of nucleotides encoding or complementary to a sequence encoding a polypeptide comprising, in its precursor form, an N-terminal signal domain, a mature domain and an acidic C-terminal domain wherein said polypeptide is produced in the

30

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epidermal layers of petals and sepals, the cortical cells of the style and the connective tissue of the anthers and its mature domain comprises the structure:-

5 a₁a₂C_Ia₃a₄a₅a₆a₇a₈a₉a₁₀a₁₁a₁₂C_{II}a₁₃a₁₄a₁₅a₁₆a₁₇C_{III}a₁₈a₁₉a₂₀C_{IV}a₂₁a₂₂a₂₃a₂₄a₂₅
a₂₆a₂₇a₂₈a₂₉C_Va₃₀a₃₁a₃₂a₃₃a₃₄a₃₅C_{VI}a₃₆C_{VII}a₃₇a₃₈a₃₉C_{VIII}

wherein "a" may be the same or different and represents any amino acid residue, the numerical subscript on each "a" represents its position in the amino acid sequence and "C" represents a cysteine residue at a position indicated by its Roman numeral and wherein the mature domain has activity against one or more plant pests with the proviso that the polypeptide is not FST or TPP3.

Still another aspect of the present invention provides an isolated nucleic acid molecule comprising a sequence of nucleotides encoding or complementary to a sequence encoding a polypeptide comprising, in its precursor form, an N-terminal signal domain, a mature domain and an acidic C-terminal domain wherein said mature domain comprises the amino acid sequence set forth in SEQ ID NO:8 or an amino acid sequence having at least about 70% similarity thereto or is encoded by a nucleotide sequence set forth in SEQ ID NO:7 or a nucleotide sequence having at least about 70% similarity thereto or a nucleotide sequence capable of hybridizing to SEQ ID NO:7 or its complementary form under low stringency conditions at 42°C.

527
25 Still a further aspect of the present invention provides a genetic construct comprising a promoter or functional equivalent thereof operably linked to a nucleotide sequence encoding a floral-derived, defensin-like molecule having a mature domain comprising the amino acid sequence:-

30 a₁a₂C_Ia₃a₄a₅a₆a₇a₈a₉a₁₀a₁₁a₁₂C_{II}a₁₃a₁₄a₁₅a₁₆a₁₇C_{III}a₁₈a₁₉a₂₀C_{IV}a₂₁a₂₂a₂₃a₂₄a₂₅
a₂₆a₂₇a₂₈a₂₉C_Va₃₀a₃₁a₃₂a₃₃a₃₄a₃₅C_{VI}a₃₆C_{VII}a₃₇a₃₈a₃₉C_{VIII}

wherein "a" may be the same or different and represents any amino acid residue, the

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numerical subscript on each "a" represents its position in the amino acid sequence and "C" represents a cysteine residue at a position indicated by its Roman numeral and wherein said mature domain exhibits inhibitory activity against plant pests such as insect pests with the proviso that the defensin-like molecule is not FST or TPP3.

5

Yet another aspect of the present invention is directed to the amino acid sequence of the mature domain comprising the amino acid sequence [SEQ ID NO:58]:-

10
$$\begin{array}{c} X_{30}X_{31}CX_{32}X_{33}X_{34}SX_{35}X_{36}FX_{37}GX_{38}CX_{39}X_{40}X_{41}X_{42}X_{43}CX_{44}X_{45}X_{46}CX_{47}X_{48}EX_{49} \\ FX_{50}X_{51}GX_{52}CX_{53}X_{54}X_{55}X_{56}X_{57}X_{58}CX_{59}CTX_{60}X_{61}C \end{array}$$

wherein	X_{30}	=	R or Q
	X_{31}	=	E, I or T
	X_{32}	=	K or E
15	X_{33}	=	T, A or S
	X_{34}	=	E, P or Q
	X_{35}	=	N, Q or H
	X_{36}	=	T or R
	X_{37}	=	P, K or H
20	X_{38}	=	I, L, P or T
	X_{39}	=	I, F, S or V
	X_{40}	=	T, M, R or S
	X_{41}	=	K, D, E or A
	X_{42}	=	P or S
25	X_{43}	=	P, S or N
	X_{44}	=	R or A
	X_{45}	=	K, T, S or N
	X_{46}	=	A, Y or V
	X_{47}	=	I, L, Q or H
30	X_{48}	=	S, K, T or N
	X_{49}	=	K or G

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	X_{50}	=	T, S, I, or V
	X_{51}	=	D or G
	X_{52}	=	H, R, or N
	X_{53}	=	S, P or R
5	X_{54}	=	K, W, A or G
	X_{55}	=	I, L or F
	X_{56}	=	L, Q, P or R
	X_{57}	=	R or P
	X_{58}	=	R or K
10	X_{59}	=	L or F
	X_{60}	=	K, S or R
	X_{61}	=	P, N or H

Yet a further aspect of the present invention is directed to the mature domain-encoding
 15 sequence operably linked to a signal domain comprising the amino acid sequence [SEQ ID
 NO:59]:-

$MX_1X_2SX_3X_4X_5X_6X_7X_8X_9X_{10}X_{11}X_{12}X_{13}X_{14}X_{15}X_{16}X_{17}X_{18}X_{19}X_{20}X_{21}X_{22}$
 $X_{23}X_{24}X_{25}X_{26}X_{27}X_{28}AX_{29}$

20

wherein

	X_1	=	A, G or K
	X_2	=	R, N, or L
	X_3	=	L, I or M
	X_4	=	C, F or R
25	X_5	=	F or L
	X_6	=	M, F or I
	X_7	=	A or S
	X_8	=	F, T or A
	X_9	=	A, L, V or F
30	X_{10}	=	I, V, L or F
	X_{11}	=	L or I

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	X ₁₂	=	A, I or M
	X ₁₃	=	M, A or F
	X ₁₄	=	M or L
	X ₁₅	=	L or I
5	X ₁₆	=	F or V
	X ₁₇	=	V, T or L
	X ₁₈	=	A, T or S
	X ₁₉	=	Y or T
	X ₂₀	=	E or G
10	X ₂₁	=	V or M
	X ₂₂	=	no amino acid or G
	X ₂₃	=	no amino acid or P
	X ₂₄	=	no amino acid, M or V
	X ₂₅	=	no amino acid or T
15	X ₂₆	=	no amino acid, I or S
	X ₂₇	=	no amino acid, A or V
	X ₂₈	=	Q or E
	X ₂₉	=	no amino acid or Q

20 Even still another aspect of the present invention is directed to the mature domain-encoding sequence operably linked to an acidic C-terminal domain comprising the sequence [SEQ ID NO:60]:-

25 X₆₂X₆₃X₆₄X₆₅X₆₆X₆₇X₆₈X₆₉X₇₀X₇₁X₇₂X₇₃X₇₄X₇₅X₇₆X₇₇X₇₈X₈₀ X₈₁X₈₂X₈₃X₈₄
X₈₅X₈₆X₈₇X₈₈X₈₉X₉₀X₉₁X₉₂X₉₃X₉₄X₉₅

wherein	X ₆₂	=	no amino acid or V
	X ₆₃	=	no amino acid or F
	X ₆₄	=	no amino acid or D
30	X ₆₅	=	no amino acid or E or K
	X ₆₆	=	no amino acid or K or I

- 10 -

	X ₆₇	=	no amino acid or M or S
	X ₆₈	=	no amino acid or T, I or S
	X ₆₉	=	no amino acid or K or E
	X ₇₀	=	no amino acid or T or V
5	X ₇₁	=	no amino acid or G or K
	X ₇₂	=	no amino acid or A
	X ₇₃	=	no amino acid or E
	X ₇₄	=	no amino acid or I or T
	X ₇₅	=	no amino acid or L
10	X ₇₆	=	no amino acid or A, V or G
	X ₇₇	=	no amino acid or E
	X ₇₈	=	no amino acid or E
	X ₇₉	=	no amino acid or A
	X ₈₀	=	no amino acid or K
15	X ₈₁	=	no amino acid or T
	X ₈₂	=	no amino acid or L
	X ₈₃	=	no amino acid or A or S
	X ₈₄	=	no amino acid or A or E
	X ₈₅	=	no amino acid or A or V
20	X ₈₆	=	no amino acid or L or V
	X ₈₇	=	no amino acid or L
	X ₈₈	=	no amino acid or E
	X ₈₉	=	no amino acid or E
	X ₉₀	=	no amino acid or E
25	X ₉₁	=	no amino acid or I
	X ₉₂	=	no amino acid or M
	X ₉₃	=	no amino acid or D or M
	X ₉₄	=	no amino acid or N or E

30 Even yet another aspect of the present invention is directed to the defensin-like molecule comprising the sequence [SEQ ID NO:61]:-

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MX₁X₂SX₃X₄X₅X₆X₇X₈X₉X₁₀X₁₁X₁₂X₁₃X₁₄X₁₅X₁₆X₁₇X₁₈X₁₉X₂₀X₂₁X₂₂X₂₃X₂₄X₂₅
X₂₆X₂₇X₂₈AX₂₉X₃₀X₃₁CX₃₂X₃₃X₃₄SX₃₅X₃₆FX₃₇GX₃₈CX₃₉X₄₀X₄₁X₄₂X₄₃CX₄₄X₄₅X₄₆C
X₄₇X₄₈EX₄₉FX₅₀X₅₁GX₅₂CX₅₃X₅₄X₅₅X₅₆X₅₇X₅₈CX₅₉CTX₆₀X₆₁CX₆₂X₆₃X₆₄X₆₅
5 X₆₆X₆₇X₆₈X₆₉X₇₀X₇₁X₇₂X₇₃X₇₄X₇₅X₇₆X₇₇X₇₈X₇₉X₈₀X₈₁X₈₂X₈₃X₈₄X₈₅X₈₆X₈₇X₈₈X₈₉X₉₀
X₉₁X₉₂X₉₃X₉₄

wherein

	X ₁	=	A, G or K
	X ₂	=	R, N, or L
10	X ₃	=	L, I or M
	X ₄	=	C, F or R
	X ₅	=	F or L
	X ₆	=	M, F or I
	X ₇	=	A or S
15	X ₈	=	F, T or A
	X ₉	=	A, L, V or F
	X ₁₀	=	I, V, L or F
	X ₁₁	=	L or I
	X ₁₂	=	A, I or M
20	X ₁₃	=	M, A or F
	X ₁₄	=	M or L
	X ₁₅	=	L or I
	X ₁₆	=	F or V
	X ₁₇	=	V, T or L
25	X ₁₈	=	A, T or S
	X ₁₉	=	Y or T
	X ₂₀	=	E or G
	X ₂₁	=	V or M
	X ₂₂	=	no amino acid or G
30	X ₂₃	=	no amino acid or P
	X ₂₄	=	no amino acid, M or V

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	X ₂₅	=	no amino acid or T
	X ₂₆	=	no amino acid, I or S
	X ₂₇	=	no amino acid or A or V
	X ₂₈	=	Q or E
5	X ₂₉	=	no amino acid or Q
	X ₃₀	=	R or Q
	X ₃₁	=	E, I or T
	X ₃₂	=	K or E
	X ₃₃	=	T, A or S
10	X ₃₄	=	E, P or Q
	X ₃₅	=	N, Q or H
	X ₃₆	=	T or R
	X ₃₇	=	P, K or H
	X ₃₈	=	I, L, P or T
15	X ₃₉	=	I, F, S or V
	X ₄₀	=	T, M, R or S
	X ₄₁	=	K, D, E or A
	X ₄₂	=	P or S
	X ₄₃	=	P, S or N
20	X ₄₄	=	R or A
	X ₄₅	=	K, T, S or N
	X ₄₆	=	A, Y or V
	X ₄₇	=	I, L, Q or H
	X ₄₈	=	S, K, T or N
25	X ₄₉	=	K or G
	X ₅₀	=	T, S, I, or V
	X ₅₁	=	D or G
	X ₅₂	=	H, R, or N
	X ₅₃	=	S, P or R
30	X ₅₄	=	K, W, A or G
	X ₅₅	=	I, L or F

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	X ₅₆	=	L, Q, P or R
	X ₅₇	=	R or P
	X ₅₈	=	R or K
	X ₅₉	=	L or F
5	X ₆₀	=	K, S or R
	X ₆₁	=	P, N or H
	X ₆₂	=	no amino acid or V
	X ₆₃	=	no amino acid or F
	X ₆₄	=	no amino acid or D
10	X ₆₅	=	no amino acid or E or K
	X ₆₆	=	no amino acid or K or I
	X ₆₇	=	no amino acid or M or S
	X ₆₈	=	no amino acid or T, I or S
	X ₆₉	=	no amino acid or K or E
15	X ₇₀	=	no amino acid or T or V
	X ₇₁	=	no amino acid or G or K
	X ₇₂	=	no amino acid or A
	X ₇₃	=	no amino acid or E
	X ₇₄	=	no amino acid or I or T
20	X ₇₅	=	no amino acid or L
	X ₇₆	=	no amino acid or A, V or G
	X ₇₇	=	no amino acid or E
	X ₇₈	=	no amino acid or E
	X ₇₉	=	no amino acid or A
25	X ₈₀	=	no amino acid or K
	X ₈₁	=	no amino acid or T
	X ₈₂	=	no amino acid or L
	X ₈₃	=	no amino acid or A or S
	X ₈₄	=	no amino acid or A or E
30	X ₈₅	=	no amino acid or A or V
	X ₈₆	=	no amino acid or L or V

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X_{87} = no amino acid or L
 X_{88} = no amino acid or E
 X_{89} = no amino acid or E
 X_{90} = no amino acid or E
5 X_{91} = no amino acid or I
 X_{92} = no amino acid or M
 X_{93} = no amino acid or D or M
 X_{94} = no amino acid or N or E

10 Another aspect of the present invention is directed to the genetic construct comprising a nucleotide sequence selected from SEQ ID NO:7, SEQ ID NO:13, SEQ ID NO:15 and SEQ ID NO:17 or a nucleotide sequence having at least 70% similarity to one or more of SEQ ID NO:8, SEQ ID NO:14, SEQ ID NO:16 and SEQ ID NO:18 or a nucleotide sequence capable of hybridizing to SEQ ID NO:7, SEQ ID NO:13, SEQ ID NO:15 and
 15 SEQ ID NO:17 or a complementary form thereof.

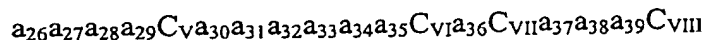
A further aspect of the present invention provides a genetic construct for use in generating insect-resistant transgenic plants, said transgenic plants producing a defensin or defensin-like molecule selected from SEQ ID NO:8, SEQ ID NO:14, SEQ ID NO:16 and SEQ ID
 20 NO:18 as well as SEQ ID NO:20 to SEQ ID NO:49 or an amino acid sequence having at least 70% similarity to any one of SEQ ID NO:8, SEQ ID NO:14, SEQ ID NO:16 and SEQ ID NO:18.

Still another aspect of the present invention further contemplates a method for generating a
 25 plant with increased or enhanced resistance to a plant pest, said method comprising introducing into the genome of a plant cell or genome of a group of plant cells a genetic construct comprising a promoter or functional equivalent thereof operably linked to a nucleotide sequence encoding a floral-derived, defensin-like molecule having a mature domain comprising the amino acid sequence:-

30

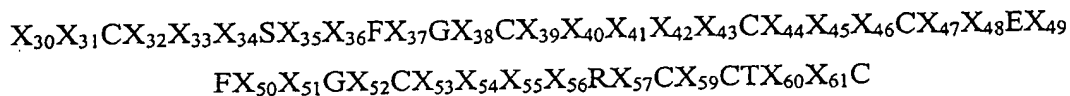
$$a_1a_2C_Ia_3a_4a_5a_6a_7a_8a_9a_{10}a_{11}a_{12}C_{II}a_{13}a_{14}a_{15}a_{16}a_{17}C_{III}a_{18}a_{19}a_{20}C_{IV}a_{21}a_{22}a_{23}a_{24}a_{25}$$

- 15 -



wherein "a" may be the same or different and represents any amino acid residue, the numerical subscript on each "a" represents its position in the amino acid sequence and "C" represents a cysteine residue at a position indicated by its Roman numeral and wherein said mature domain exhibits inhibitory activity against plant pests such as insect pests and regenerating a plant from said cell or group of cells.

Still a further aspect of the present invention is directed to the defensin-like molecule comprising a mature domain having the amino acid sequence [SEQ ID NO:58]:-



15	wherein	X_{30}	=	R or Q
		X_{31}	=	E, I or T
		X_{32}	=	K or E
		X_{33}	=	T, A or S
		X_{34}	=	E, P or Q
20		X_{35}	=	N, Q or H
		X_{36}	=	T or R
		X_{37}	=	P, K or H
		X_{38}	=	I, L, P or T
		X_{39}	=	I, F, S or V
25		X_{40}	=	T, M, R or S
		X_{41}	=	K, D, E or A
		X_{42}	=	P or S
		X_{43}	=	P, S or N
		X_{44}	=	R or A
30		X_{45}	=	K, T, S or N
		X_{46}	=	A, Y or V

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	X ₄₇	=	I, L, Q or H
	X ₄₈	=	S, K, T or N
	X ₄₉	=	K or G
	X ₅₀	=	T, S, I, or V
5	X ₅₁	=	D or G
	X ₅₂	=	H, R, or N
	X ₅₃	=	S, P or R
	X ₅₄	=	K, W, A or G
	X ₅₅	=	I, L or F
10	X ₅₆	=	L, Q, P or R
	X ₅₇	=	R or P
	X ₅₈	=	R or K
	X ₅₉	=	L or F
	X ₆₀	=	K, S or R
15	X ₆₁	=	P, N or H

545
67 Yet another aspect of the present invention provides a method for generating a plant with increased or enhanced resistance to an insect, said method comprising introducing into the genome of a plant cell or genome of a group of plant cells a genetic construct comprising a promoter or functional equivalent thereof operably linked to a nucleotide sequence encoding a defensin-like molecule having a mature domain comprising the amino acid sequence:-

25 a₁a₂C_Ia₃a₄a₅a₆a₇a₈a₉a₁₀a₁₁a₁₂C_{II}a₁₃a₁₄a₁₅a₁₆a₁₇C_{III}a₁₈a₁₉a₂₀C_{IV}a₂₁a₂₂a₂₃a₂₄a₂₅
a₂₆a₂₇a₂₈a₂₉C_Va₃₀a₃₁a₃₂a₃₃a₃₄a₃₅C_{VI}a₃₆C_{VII}a₃₇a₃₈a₃₉C_{VIII}

wherein "a" may be the same or different and represents any amino acid residue, the numerical subscript on each "a" represents its position in the amino acid sequence and "C" represents a cysteine residue at a position indicated by its Roman numeral and wherein said mature domain exhibits inhibitory activity against plant pests such as insect pests and regenerating a plant from said cell or group of cells.

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Yet a further aspect of the present invention provides a transfected or transformed cell, tissue or organ from a plant or a transformed microbial cell, said cell, tissue or organ comprising a nucleic acid molecule comprising a sequence of nucleotides encoding or complementary to a sequence encoding a polypeptide comprising, in its precursor form, an N-terminal signal domain, a mature domain and an acidic C-terminal domain wherein said polypeptide is produced during flower development and its mature domain has activity against one or more plant pests.

10 ~~Sub Ag~~ Even still another aspect of the present invention is directed to the nucleic acid molecule comprising a sequence of nucleotides encoding or complementary to a sequence encoding a polypeptide comprising, in its precursor form, an N-terminal signal domain, a mature domain and an acidic C-terminal domain wherein said polypeptide is produced during flower development and its mature domain comprises the structure:-

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$$a_1a_2C_Ia_3a_4a_5a_6a_7a_8a_9a_{10}a_{11}a_{12}C_{II}a_{13}a_{14}a_{15}a_{16}a_{17}C_{III}a_{18}a_{19}a_{20}C_{IV}a_{21}a_{22}a_{23}a_{24}a_{25}$$
$$a_{26}a_{27}a_{28}a_{29}C_Va_{30}a_{31}a_{32}a_{33}a_{34}a_{35}C_{VI}a_{36}C_{VII}a_{37}a_{38}a_{39}C_{VIII}$$

20 wherein "a" may be the same or different and represents any amino acid residue, the numerical subscript on each "a" represents its position in the amino acid sequence and "C" represents a cysteine residue at a position indicated by its Roman numeral and wherein the mature domain has activity against one or more plant pests.

25 Even yet another aspect of the present invention, insofar as it relates to plants, further extends to progeny of the plants engineered to express the nucleic acid molecule encoding the defensin-like molecule or a variant or homologue thereof as well as vegetative, propagative and reproductive parts of the plants, such as flowers (including cut or severed flowers), parts of plants, fibrous material from plants (for example, cotton) and reproductive portions including cuttings, pollen, seeds and callus.

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Another aspect of the present invention provides a genetically modified plant cell or

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multicellular plant or progeny thereof or parts of a genetically modified plant capable of producing a heterologous defensin-like molecule as herein described wherein said transgenic plant is resistant or has reduced sensitivity to plant pests such as insects.

- 5 A further aspect of the present invention comprises one or more genetic constructs alone or in combination comprising a first promoter operably linked to a first nucleotide sequence wherein said first nucleotide sequence encodes a defensin-like molecule capable of inhibiting a plant pest such as an insect, said construct further comprising a second promoter operably linked to a second nucleotide sequence wherein said second nucleotide
- 10 sequence encodes a proteinase inhibitor or precursor thereof.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is a representation of the nucleotide sequence (SEQ ID NO:17) and the predicted amino acid sequence (SEQ ID NO:18) of *NaPdf1* (*Nicotiana alata* plant defensin 1), the cDNA encoding the floral defensin from *Nicotiana alata*. Only one strand with the polarity of the mRNA is shown and the nucleotides are numbered above. The amino acid sequence, shown in single letter code, is given below the nucleotide sequence and is numbered beginning with 1 for the first amino acid of the mature protein. The putative signal peptide is indicated by negative numbers and is underlined. The mature protein is highlighted and arrows depict the predicted cleavage sites of the signal peptide and the end of the mature protein. The first stop codon is marked with an asterisk (*) and the two polyadenylation sites are in bold. For further detail, refer to Example 7.

Figure 2 is a diagrammatic representation of an RNA gel blot analysis of *NaPdf1* expression in various tissues of *Nicotiana alata*. Total RNA was isolated from anthers at stages I (5 - 10 mm buds), II (20 - 30 mm buds) and III (50 - 70 mm buds) of development, from pollen grains, and from mature pistil, ovary, petal, leaf and root tissues of *N. alata* (self-incompatibility genotype, S_2S_2), as shown in panel A. Panel B shows the same RNA samples following staining with ethidium bromide.

Figure 3 is a representation of an autoradiograph showing *in situ* localization of *NaPdf1* RNA. (A) A transverse section of a 1 cm long flower bud, after hybridization with a ^{35}S -labelled *NaPdf1* anti-sense RNA probe. Heavy labelling of the epidermal cells of the petal (Pe) and sepal (Se), the cortical cells of the pistil (Pi) and the connective tissue of the anther (A) can be detected. (B) The same section as in A, under higher magnification. (C) A similar section as in B, after hybridization with a ^{35}S -labelled *NaPdf1* sense RNA probe. No labelling is seen in any of the cells of the pistil (Pi), anther (A), petal (Pe) or sepal (Se).

Figure 4A is a representation showing bacterial expression of N-terminal hexahistidine-tagged pro-defensin (6H.NaproPdf1) encoded by the *NaPdf1* cDNA. Lane 1: total protein extracted 6 h post-induction with 1 mM IPTG in 1xSDS sample loading buffer. Lane 2:

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soluble proteins in the 8 M urea lysate. Lane 3: induced protein purified by IMAC. The proteins were separated on a 15% w/v SDS-polyacrylamide gel and were stained with Coomassie Blue. Molecular size markers are the Broad Range standards from Bio-Rad. The induced ~12 kDa protein (arrowed) was substantially pure after immobilized metal
5 affinity chromatography (IMAC).

Figure 4B shows a reverse-phase HPLC chromatogram of a sample of the metal affinity purified 6H.NaproPdf1 protein (IMAC from A), eluted as described in Example 5.

10 **Figure 5** is a representation showing immunoblot analysis of plant extracts with the antibodies raised to the bacterially expressed pro-defensin (6H.NaproPdf1) encoded by the *NaPdf1* cDNA clone. (A) A diagrammatic representation of the five stages of developing *N. alata* flowers. (B) A representation of an immunoblot of buffer soluble proteins (60 µg)
15 w/v SDS-polyacrylamide gel prior to transfer to nitrocellulose (0.22 µm) and immunoblotting with antibodies (1:2500) raised against bacterially expressed 6H.NaproPdf1 (see Figure 4). The antibodies bound specifically to three proteins. The smallest is the predicted size of mature defensin (~5 kDa) while the two larger species are probably the precursor and a processing intermediate. For details, see Examples 4 and 5.

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Figure 6 shows the purification of mature *N. alata* defensin from flower buds. (A) Reverse-phase HPLC on an Aquapore RP-300 C8 column (4.6 mm x 100 mm, Brownlee). Proteins were extracted from flower buds and partially purified by gel filtration chromatography (see Example 5) before RP-HPLC. Proteins were applied in 0.1% v/v
25 TFA and eluted with 60% v/v acetonitrile in 0.089% v/v TFA (buffer B) according to the gradient 0-100% buffer B over 40 min at a flow rate of 1 mL/min. Eluted proteins were detected by absorbance at 215 nm. (Inset) Protein in Peak A separated by 15% w/v SDS-PAGE and immunoblotted with anti-6H.NaproPdf1 antibodies (Example 4). (B) N-terminal sequencing and mass spectrometry confirmed the identity of Peak A as the mature
30 defensin domain encoded by the *NaPdf1* cDNA clone. "x" corresponds to an unassigned amino acid that is probably a cysteine as predicted from the cDNA sequence

Figure 7 is a series of electron micrographs showing the location of the *N. alata* defensin in anthers and ovaries from 10 mm flower buds. (A) Overview of the anther showing the cells of the connective tissue with electron dense deposits (arrowed) in the vacuole. (B) Immunogold localization of the defensin in the cells of the connective tissue of the anther. The antibody bound to the electron dense deposits in the vacuole (v) and did not bind to the cytoplasm (ct) or the cell wall (cw). (C) Immunogold localization of the defensin in the cortical cells of the ovary. The antibody bound specifically to electron dense deposits in the vacuole and no binding was observed in the cytoplasm or cell walls.

Figure 8 is a schematic representation of the precursor proteins predicted from cDNA clones that encode floral and seed defensins. (A) Some floral defensins are produced as precursor proteins with three distinct domains: an ER signal sequence (left section of the diagram), a central basic domain (middle of the diagram) and a C-terminal domain rich in acidic amino acids (right section of the diagram). The predicted sizes of each of these domains for the *N. alata* defensin are shown below the diagram. The mature floral defensin is released after proteolytic cleavage (arrowed). (B) cDNAs for seed derived defensins encode proteins with an ER signal sequence and a basic defensin domain, but no C-terminal acidic domain.

Figure 9 is an alignment of the amino acid sequence of NaPdf1 with the predicted amino acid sequences encoded from five other flower-derived cDNA clones, as follows:

FST

(flower specific thionin):

Gu *et al.*, *Mol. Gen. Genet.* 234: 89-96, 1992;

TPP3:

Milligan and Gasser, *Plant Mol. Biol.* 28: 691-711, 1995;

NTS13:

Li and Gray, *Plant Physiology* 120: 633, 1999;

PPT:

Karunanandaa *et al.*, *Plant Mol. Biol.*, 26: 459-464, 1994;

ATPIIIa:

Yu *et al.*, Direct Submission, Accession No. S30578, 1999.

Some, but not all floral defensins have a C-terminal acidic domain of 32-33 amino acids.

Figure 10 is an alignment of the amino acid sequence of the mature domain of NaPdfl with the amino acid sequences of the mature domain of other members of the plant defensin family. The N-terminal amino acid in the Rs-AFP1, Rs-AFP2, M1, M2A and M2B sequence which is represented by "pQ" is a pyroglutamic acid. The sequences are derived from the following sources:-

	FST:	Gu <i>et al.</i> (1992; <i>supra</i>) (SEQ ID NO:25);
	TPP3:	Milligan and Gasser (1995; <i>supra</i>) (SEQ ID NO:26);
10	p322:	Steikema <i>et al.</i> , <i>Plant Mol. Biol.</i> 11: 255-269, 1988 (SEQ ID NO:27);
	PPT:	Karunanandaa <i>et al.</i> (1994; <i>supra</i>) (SEQ ID NO:28);
	SE60:	Choi <i>et al.</i> , <i>Plant Physiology</i> 101: 699-700, 1993; Choi <i>et al.</i> , <i>Mol. Gen. Genet.</i> 246: 266-268, 1995 (SEQ ID NO:29);
15	γ 1-H:	Mendez <i>et al.</i> , <i>Eur. J. Biochem.</i> 194: 533-539, 1990 (SEQ ID NO:30);
	M2A, M1 and M2B:	Neumann <i>et al.</i> , <i>Int. J. Protein & Peptide Research</i> 47: 437-446, 1996 (SEQ ID NO:31, SEQ ID NO:35 and SEQ ID NO:36, respectively);
20	PTH-St1:	Moreno <i>et al.</i> , <i>Eur. J. Biochem.</i> 223: 135-139, 1995 (SEQ ID NO:32);
	Rs-AFP1 and Rs-AFP2:	Terras <i>et al.</i> , <i>J. Biological Chemistry</i> 267: 15301-15309, 1992; Terras <i>et al.</i> , <i>FEBS Letters</i> 316: 233-240, 1993; Terras <i>et al.</i> , <i>Plant Cell</i> 7: 573-588, 1995; and Fant <i>et al.</i> , <i>The solution structure by ¹H-NMR of Rs-AFP1, a plant antifungal protein from radish seeds</i> . In: LP Ingman, J Jokissaari, J Lounila (eds), <i>Abstracts of the 12th European Experimental NMR Conference</i> , p 247, 1994 (SEQ ID NO:33 and SEQ ID NO:34, respectively);
25		
30	γ 1-P:	Collila <i>et al.</i> , <i>FEBS Letters</i> 270: 191-194, 1990 (SEQ ID NO:37);

- γ 2-P: Collila *et al.*, (1990; *supra*) (SEQ ID NO:38);
- 10kDa: Ishibashi *et al.*, *Plant Mol. Biol.* 15: 59-64, 1992 (SEQ ID NO:39);
- 5 SI α 2, SI α 3 and SI α 1: Bloch and Richardson, *FEBS Letters* 279: 101-104, 1991 and Nitti *et al.*, *Eur. J. Biochem.* 228: 250-256, 1995 (SEQ ID NO:40, SEQ ID NO:41 and SEQ ID NO:43, respectively);
- Dm-AMP2, Ah-AMP1, Hs-AFP1, Dm-AMP1 and
- 10 Ct-AMP1: Osborn *et al.*, *FEBS Letters* 368: 257-262, 1995 (SEQ ID NO:42, SEQ ID NO:45, SEQ ID NO:46, SEQ ID NO:47 and SEQ ID NO:49, respectively);
- pI230 and P139: Chiang and Hagwiger, *Mol. Plant-Microbe Interact.* 4: 324-331, 1991 (SEQ ID NO:44 and SEQ ID NO:48, respectively);
- 15 NeThio1 and NeThio2: Yamada *et al.*, *Plant Physiology* 115: 314, 1997; (SEQ ID NO:50 and SEQ ID NO:51); and
- NpThio1: Komori *et al.*, *Plant Physiology* 115: 314, 1997 (SEQ ID NO:52).

20

Figure 11 shows growth inhibition curves of various agents against *Botrytis cinerea*, as monitored by absorbance at 595 nm. Each treatment was performed in quadruplicate. Purified NaPdf1 protein at 20 μ g/ml was assayed. Water and ovalbumin (20 μ g/ml) served as negative controls and a mixture of the antifungal proteins α - and β -purothionin (20 μ g/ml) was used as a positive control.

25

Figure 12 shows growth inhibition curves of various agents against *Fusarium oxysporum f. sp. dianthi* (A) and *F. oxysporum f. sp. vasinfectum* (B and C), as monitored by absorbance at 595 nm. Each treatment was performed in quadruplicate. Purified NaPdf1 protein at 20 μ g/ml (A and B) and 10 μ g/ml (C) were assayed. Water and ovalbumin (20 μ g/ml, A and B; 10 μ g/ml, C) served as negative controls, and a mixture of the antifungal

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proteins α - and β -purothionin (20 μ g/ml, A and B; 10 μ g/ml, C) was used as a positive control.

Figure 13 is a schematic of plant transformation constructs pFL1 and pHEX3, used for the transformation of tobacco and cotton. Both constructs contain the *N. alata* defensin, NaPdfl, under the control of the CaMV35S promoter/terminator. The region designated "surB" codes for resistance to the herbicide glean, and that designated "nptII" codes for resistance against the antibiotic kanamycin.

Figure 14 shows representations of protein blots indicating expression of (A) *N. alata* proteinase inhibitor (NaPI) protein and (B) NaPdfl protein in transgenic tobacco plants. In A, lane 1: 25ng NaPI; lane 2: 100 ng NaPI; lane 3: pHEX3.4; lane 4: untransformed W38 and lane 5: pFL1/W19. In B, lane 1: pHEX3.4; lane 2: pFL1/W19; lane 3: untransformed W38 and lane 4: *N. alata* bud extract. (C) indicates expression of NaPdfl protein in a transgenic cotton plant. Lane 1: 25 ng purified NaPdfl; lane 2: plant CT28.14.1 (transformed with unrelated plasmid) and lane 3: plant CT35.9.1 (transformed with pHEX3).

Figure 15 shows growth curves for *H. punctigera* and *H. armigera* fed on transgenic *N. tabacum* leaves (lines pHEX3.4 and pFL1/W19) transformed with the NaPdfl gene and an untransformed W38 parent plant. (A) Survival of *H. punctigera* larvae, measured between days 2 and 18, (B) the average mean weight of *H. punctigera* larvae measured between days 7 and 18, (C) survival of *H. armigera* larvae measured between days 3 and 23, (D) the average mean weight of *H. armigera* larvae measured between days 6 and 23.

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Figure 16 shows the average mean weight of *H. armigera* larvae fed on artificial diet containing either 0.03% NaPdfl, 0.3% NaPdfl, 0.3% NaPI or casein in place of the test protein (control). Weight of larvae was measured after 6, 9, 12 and 14 days of feeding.

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Figure 17 shows the average mean weight of *H. armigera* larvae fed on transgenic cotton (lines CT35.9.4 and CT35.125.1) transformed with NaPdf1 and non-transformed parent Coker 315 at day 8.

- 5 Table 1 is a summary of amino acid and nucleotide sequence identifiers.

Table 2 Artificial diet ingredients used in the feeding trial of *H. armigera*

TABLE 1

SEQUENCE ID NO:	DESCRIPTION
SEQ ID NO:1	Primer FST1
SEQ ID NO:2	Primer FST2
SEQ ID NO:3	Primer PDF1
SEQ ID NO:4	Primer PDF2
SEQ ID NO:5	Primer FLOR1
SEQ ID NO:6	Primer FLOR2
SEQ ID NO:7	cDNA encoding mature domain (NaPdf1)
SEQ ID NO:8	Amino acid sequence corresponding to SEQ ID NO:7
SEQ ID NO:9	cDNA encoding N-terminal domain (NaPdf1)
SEQ ID NO:10	Amino acid sequence corresponding to SEQ ID NO:9
SEQ ID NO:11	cDNA encoding C-terminal acidic tail (NaPdf1)
SEQ ID NO:12	Amino acid sequence corresponding to SEQ ID NO:11
SEQ ID NO:13	cDNA encoding N-terminal + mature domain (NaPdf1)
SEQ ID NO:14	Amino acid sequence corresponding to SEQ ID NO:13
SEQ ID NO:15	cDNA encoding mature + acidic C-terminal domain (NaPdf1)
SEQ ID NO:16	Amino acid sequence corresponding to SEQ ID NO:15
SEQ ID NO:17	cDNA encoding NaPdf1
SEQ ID NO:18	Amino acid sequence corresponding to SEQ ID NO:17
SEQ ID NO:19	cDNA corresponding to 3' end of NaPdf1
SEQ ID NO:20	Amino acid sequence of full FST
SEQ ID NO:21	Amino acid sequence of full TPP3
SEQ ID NO:22	Amino acid sequence of full NTS13
SEQ ID NO:23	Amino acid sequence of full PPT
SEQ ID NO:24	Amino acid sequence of full ATP11a
SEQ ID NO:25	Amino acid sequence of mature domain of FST
SEQ ID NO:26	Amino acid sequence of mature domain of TPP3
SEQ ID NO:27	Amino acid sequence of mature domain of P322

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SEQUENCE ID NO:	DESCRIPTION
SEQ ID NO:28	Amino acid sequence of mature domain of PPT
SEQ ID NO:29	Amino acid sequence of mature domain of SE60
SEQ ID NO:30	Amino acid sequence of mature domain of γ 1-H
SEQ ID NO:31	Amino acid sequence of mature domain of M2A
SEQ ID NO:32	Amino acid sequence of mature domain of PTH-St1
SEQ ID NO:33	Amino acid sequence of mature domain of Rs-AFP1
SEQ ID NO:34	Amino acid sequence of mature domain of Rs-AFP2
SEQ ID NO:35	Amino acid sequence of mature domain of M1
SEQ ID NO:36	Amino acid sequence of mature domain of M2B
SEQ ID NO:37	Amino acid sequence of mature domain of γ 1-P
SEQ ID NO:38	Amino acid sequence of mature domain of γ 2-P
SEQ ID NO:39	Amino acid sequence of mature domain of 10 kDa
SEQ ID NO:40	Amino acid sequence of mature domain of SI α 2
SEQ ID NO:41	Amino acid sequence of mature domain of SI α 3
SEQ ID NO:42	Amino acid sequence of mature domain of Dm-AMP2
SEQ ID NO:43	Amino acid sequence of mature domain of SI α 1
SEQ ID NO:44	Amino acid sequence of mature domain of P1230
SEQ ID NO:45	Amino acid sequence of mature domain of Ah-AMP1
SEQ ID NO:46	Amino acid sequence of mature domain of Hs-AFP1
SEQ ID NO:47	Amino acid sequence of mature domain of Dm-AMP1
SEQ ID NO:48	Amino acid sequence of mature domain of P139
SEQ ID NO:49	Amino acid sequence of mature domain of Ct-AMP1
SEQ ID NO:50	Amino acid sequence of mature domain of NeThio1
SEQ ID NO:51	Amino acid sequence of mature domain of NeThio2
SEQ ID NO:52	Amino acid sequence of mature domain of NpThio1
SEQ ID NO:53	Amino acid sequence of mature domain of NTS13
SEQ ID NO:54	Amino acid sequence of mature domain of PPT
SEQ ID NO:55	Amino acid sequence of mature domain of ATPIIIa
SEQ ID NO:56	cDNA encoding mature domain of NaPI

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SEQUENCE ID NO:	DESCRIPTION
SEQ ID NO:57	Amino acid sequence corresponding to SEQ ID NO:56
SEQ ID NO:58	Consensus sequence of mature domain of defensin
SEQ ID NO:59	Consensus sequence of internal domain of defensin
SEQ ID NO:60	Consensus sequence of C-terminal domain of defensin
SEQ ID NO:61	Consensus amino acid sequence of defensin

2498045.bcx\ms.defensin.us.doc-08/02/02

TABLE 2

Reagent	Control (10 g)	0.3% NaPdl (3 g)	0.03% NaPdl (3 g)	0.3% NaPI (3 g)
Powdered cotton leaf	300 mg	90 mg	90 mg	90 mg
Yeast	200 mg	60 mg	60 mg	60 mg
Wheatgerm	240 mg	72 mg	72 mg	72 mg
Ascorbic acid	320 mg	96 mg	96 mg	96 mg
Sorbic acid	8 mg	2.4 mg	2.4 mg	2.4 mg
Paraben	16 mg	4.8 mg	4.8 mg	4.8 mg
Linseed oil	8 µl	2.4 µl	2.4 µl	2.4 µl
Wheatgerm oil	16 µl	4.8 µl	4.8 µl	4.8 µl
Casein	26.5 mg	-	7.155 mg	-
Inhibitor protein (15 mg/ml)	-	530 µl	53 µl	600 µl
Distilled water	1.66 ml	-	444 µl	-
The above reagents were mixed and then added to melted agar				
Agar	320 mg	96 mg	96 mg	96 mg
Distilled water	6 ml	1.77 ml	1.8 ml	1.7 ml
Add and mix				
Ampicillin (200 mg/ml)	14 µl	4.2 µl	4.2 µl	4.2 µl
Streptomycin (200mg/ml)	14 µl	4.2 µl	4.2 µl	4.2 µl

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention is predicated in part on the determination of the biological properties of floral-derived, defensin-like molecules from plants and the elucidation of new properties in seed-derived defensins and previously known floral-derived defensins. Importantly, a novel floral-derived, defensin-like molecule is described which exhibits activity against plant pathogens and in particular plant pests such as insects and fungi. Other defensin molecules are described which are contemplated to exhibit anti-insect activity.

Accordingly, one aspect of the present invention provides an isolated nucleic acid molecule comprising a sequence of nucleotides encoding or complementary to a sequence encoding a polypeptide comprising, in its precursor form, an N-terminal signal domain, a mature domain and an acidic C-terminal domain wherein said polypeptide is produced during flower development and its mature domain has activity against one or more plant pests.

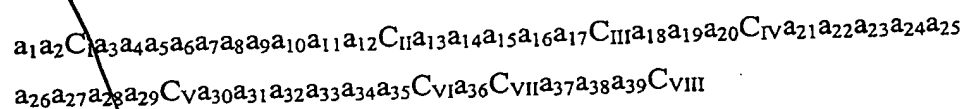
This aspect of the present invention does not extend to the defensins FST [flower specific thionin] (Gu *et al.*, {1992; *supra*}) or TPP3 (Milligan and Gasser, {1995; *supra*}).

Reference herein to a "polypeptide" includes reference to a peptide or protein. Generally, the polypeptide comprises cysteine residues, the location of which is conserved within members of floral and non-floral-derived defensin molecules. The location of the eight cysteine residues may be defined as follows:-

a₁a₂C_Ia₃a₄a₅a₆a₇a₈a₉a₁₀a₁₁a₁₂C_{II}a₁₃a₁₄a₁₅a₁₆a₁₇C_{III}a₁₈a₁₉a₂₀C_{IV}a₂₁a₂₂a₂₃a₂₄a₂₅
a₂₆a₂₇a₂₈a₂₉C_Va₃₀a₃₁a₃₂a₃₃a₃₄a₃₅C_{VI}a₃₆C_{VII}a₃₇a₃₈a₃₉C_{VIII}

wherein "a" may be the same or different and represents any amino acid residue, the numerical subscript on each "a" represents its position in the amino acid sequence and "C" represents a cysteine residue at a position indicated by its Roman numeral.

Accordingly, another aspect of the present invention is directed to an isolated nucleic acid molecule comprising a sequence of nucleotides encoding or complementary to a sequence encoding a polypeptide comprising, in its precursor form, an N-terminal signal domain, a mature domain and an acidic C-terminal domain wherein said polypeptide is produced during flower development and its mature domain comprises the structure:-



wherein "a" may be the same or different and represents any amino acid residue, the numerical subscript on each "a" represents its position in the amino acid sequence and "C" represents a cysteine residue at a position indicated by its Roman numeral and wherein the mature domain has activity against one or more plant pests with the proviso that the polypeptide is not FST or TPP3.

The term "isolated" means that the nucleic acid molecule has undergone at least one step towards being isolated or concentrated or enriched from a more complex solution or source. For example, the term "isolated" includes nucleic acid molecules concentrated or enriched from a biological or chemical sample by precipitation, centrifugation, electrophoresis, micro-filtration, electroporation or chromatography. The term "isolated", however, is in no way intended to limit the nucleic acid molecule to a particular location or state and the present invention extends to the nucleic acid molecule when introduced into the genome of a cell or when it is resident in progeny of cells into which the nucleic acid molecule has been introduced into its genome.

Reference herein to a "nucleic acid molecule" includes reference to DNA or RNA (e.g. mRNA) or DNA/RNA hybrids. A nucleic acid molecule may be regarded *inter alia* as a genetic molecule, nucleotide sequence or polynucleotide sequence. Preferably, the nucleic acid molecule is a cDNA molecule although the present invention extends to genomic forms of the nucleic acid molecule. The nucleic acid molecule of the present invention may also encode separately the N-terminal signal domain, the mature domain and/or the acidic

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C-terminal domain or combinations thereof. For example, the nucleic acid molecule may encode for the N-terminal signal domain operably linked to the mature domain. Alternatively, it may encode for the mature domain operably linked to the acidic C-terminal domain. The nucleic acid molecule may also encode all three domains or
 5 comprise heterologous domains from other defensin or defensin-like molecules. The development of heterologous defensin-like molecules is encompassed in the present invention and provides a means of broadening the anti-insect or anti-pest spectrum. A heterologous molecule may also comprise multiple mature domains and random repeats of mature domains or other domains required for activity.

10

Reference herein to production of the polypeptide during "flower development" includes reference to production in flowering parts such as but not limited to production in the pistils, anthers, ovaries, sepals and petals of the flowering region. Preferably, the polypeptide is produced in the epidermal layers of the petals and sepals, the cortical cells of
 15 the style and the connective tissue of the anthers.

Accordingly, another aspect of the present invention contemplates an isolated nucleic acid molecule comprising a sequence of nucleotides encoding or complementary to a sequence encoding a polypeptide comprising, in its precursor form, an N-terminal signal domain, a
 20 mature domain and an acidic C-terminal domain wherein said polypeptide is produced in the epidermal layers of petals and sepals, the cortical cells of the style and connective tissue of the anthers and its mature domain comprises the structure:-

25 $a_1 a_2 C_I a_3 a_4 a_5 a_6 a_7 a_8 a_9 a_{10} a_{11} a_{12} C_{II} a_{13} a_{14} a_{15} a_{16} a_{17} C_{III} a_{18} a_{19} a_{20} C_{IV} a_{21} a_{22} a_{23} a_{24} a_{25}$
 $a_{26} a_{27} a_{28} a_{29} C_V a_{30} a_{31} a_{32} a_{33} a_{34} a_{35} C_{VI} a_{36} C_{VII} a_{37} a_{38} a_{39} C_{VIII}$

wherein "a" may be the same or different and represents any amino acid residue, the numerical subscript on each "a" represents its position in the amino acid sequence and "C" represents a cysteine residue at a position indicated by its Roman numeral and wherein the
 30 mature domain has activity against one or more plant pests with the proviso that the polypeptide is not FST or TPP3.

In an alternative embodiment, the present invention provides an isolated nucleic acid molecule comprising a sequence of nucleotides encoding or complementary to a sequence encoding a polypeptide comprising two or more mature domains having activity against one or more plant pests and optionally an N-terminal signal domain and optionally an acidic C-terminal domain. The polypeptide according to the latter embodiment would be regarded as a fusion polypeptide.

The location of production of the subject polypeptide may be altered depending on the genetic construct employed to express the nucleic acid molecule. For example, a developmentally regulated and/or tissue-specific promoter may be employed to direct expression of the nucleic acid molecule in any tissue. However, the naturally occurring polypeptide is produced during flower development and more particularly in the tissues of flowers outlined above.

The term "plant pests" is not to confer any limitation as to the type of organism targeted by the subject defensin-like molecule. A plant pest includes an insect, arachnid, microorganism, fungus or virus. In a particularly preferred embodiment, the plant pest is an insect.

In a most preferred embodiment, the defensin-like molecule or its encoding nucleic acid molecule is isolatable from *N. alata* and related species or varieties or strains thereof. The amino acid sequence of the mature domain of the *N. alata* defensin is as follows (in single letter code):

RECKTESNTF PGICITKPPC RKACISEKFT DGHCSKILRR CLCTKPC [SEQ ID NO:8].

The nucleotide sequence encoding SEQ ID NO:8 is set forth in SEQ ID NO:7.

The present invention extends to novel variants of SEQ ID NO:8 such as variants with an

amino acid sequence having at least 70% similarity to the sequence set forth in SEQ ID NO:8 or variants encoded by a nucleotide sequence capable of hybridizing to the nucleotide sequence encoding SEQ ID NO:8 (i.e. SEQ ID NO:7) under low stringency conditions at 42°C.

5

Accordingly, another aspect of the present invention provides an isolated nucleic acid molecule comprising a sequence of nucleotides encoding or complementary to a sequence encoding a polypeptide comprising, in its precursor form, an N-terminal signal domain, a mature domain and an acidic C-terminal domain wherein said mature domain comprises
10 the amino acid sequence set forth in SEQ ID NO:8 or an amino acid sequence having at least about 70% similarity thereto or is encoded by a nucleotide sequence set forth in SEQ ID NO:7 or a nucleotide sequence having at least about 70% similarity thereto or a nucleotide sequence capable of hybridizing to SEQ ID NO:7 or its complementary form under low stringency conditions at 42°C.

15

The term "similarity" as used herein includes exact identity between compared sequences at the nucleotide or amino acid level. Where there is non-identity at the nucleotide level, "similarity" includes differences between sequences which result in different amino acids that are nevertheless related to each other at the structural, functional, biochemical and/or
20 conformational levels. Where there is non-identity at the amino acid level, "similarity" includes amino acids that are nevertheless related to each other at the structural, functional, biochemical and/or conformational levels. In a particularly preferred embodiment, nucleotide and sequence comparisons are made at the level of identity rather than similarity.

25

Terms used to describe sequence relationships between two or more polynucleotides or polypeptides include "reference sequence", "comparison window", "sequence similarity", "sequence identity", "percentage of sequence similarity", "percentage of sequence identity", "substantially similar" and "substantial identity". A "reference sequence" is at
30 least 12 but frequently 15 to 18 and often at least 25 or above, such as 30 monomer units, inclusive of nucleotides and amino acid residues, in length. Because two polynucleotides

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may each comprise (1) a sequence (i.e. only a portion of the complete polynucleotide sequence) that is similar between the two polynucleotides, and (2) a sequence that is divergent between the two polynucleotides, sequence comparisons between two (or more) polynucleotides are typically performed by comparing sequences of the two polynucleotides over a "comparison window" to identify and compare local regions of sequence similarity. A "comparison window" refers to a conceptual segment of typically 12 contiguous residues that is compared to a reference sequence. The comparison window may comprise additions or deletions (i.e. gaps) of about 20% or less as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. Optimal alignment of sequences for aligning a comparison window may be conducted by computerised implementations of algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package Release 7.0, Genetics Computer Group, 575 Science Drive Madison, WI, USA) or by inspection and the best alignment (i.e. resulting in the highest percentage homology over the comparison window) generated by any of the various methods selected. Reference also may be made to the BLAST family of programs as, for example, disclosed by Altschul *et al.* (*Nucl. Acids Res.* 25: 3389, 1997). A detailed discussion of sequence analysis can be found in Unit 19.3 of Ausubel *et al.* ("Current Protocols in Molecular Biology" John Wiley & Sons Inc, 1994-1998, Chapter 15).

The terms "sequence similarity" and "sequence identity" as used herein refers to the extent that sequences are identical or functionally or structurally similar on a nucleotide-by-nucleotide basis or an amino acid-by-amino acid basis over a window of comparison. Thus, a "percentage of sequence identity", for example, is calculated by comparing two optimally aligned sequences over the window of comparison, determining the number of positions at which the identical nucleic acid base (e.g. A, T, C, G, I) or the identical amino acid residue (e.g. Ala, Pro, Ser, Thr, Gly, Val, Leu, Ile, Phe, Tyr, Trp, Lys, Arg, His, Asp, Glu, Asn, Gln, Cys and Met) occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison (i.e., the window size), and multiplying the result by 100 to yield the percentage of sequence identity. For the purposes of the present invention, "sequence

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identity" will be understood to mean the "match percentage" calculated by the DNASIS computer program (Version 2.5 for windows; available from Hitachi Software engineering Co., Ltd., South San Francisco, California, USA) using standard defaults as used in the reference manual accompanying the software. Similar comments apply in relation to
5 sequence similarity.

Reference herein to a "low stringency" includes and encompasses from at least about 0 to at least about 15% v/v formamide and from at least about 1 M to at least about 2 M salt for hybridization, and at least about 1 M to at least about 2 M salt for washing conditions.
10 Generally, low stringency is from about 25-30°C to about 42°C. The temperature may be altered and higher temperatures used to replace formamide and/or to give alternative stringency conditions. Alternative stringency conditions may be applied where necessary, such as medium stringency, which includes and encompasses from at least about 16% v/v to at least about 30% v/v formamide and from at least about 0.5 M to at least about 0.9 M
15 salt for hybridization, and at least about 0.5 M to at least about 0.9 M salt for washing conditions, or high stringency, which includes and encompasses from at least about 31% v/v to at least about 50% v/v formamide and from at least about 0.01 M to at least about 0.15 M salt for hybridization, and at least about 0.01 M to at least about 0.15 M salt for washing conditions. In general, washing is carried out $T_m = 69.3 + 0.41 (G+C)\%$ (Marmur and Doty, *J. Mol. Biol.* 5: 109, 1962). However, the T_m of a duplex DNA decreases by 1°C with every increase of 1% in the number of mismatch base pairs (Bonner and Laskey, *Eur. J. Biochem.* 46: 83, 1974). Formamide is optional in these hybridization conditions. Accordingly, particularly preferred levels of stringency are defined as follows: low stringency is 6 x SSC buffer, 0.1% w/v SDS at 25-42°C; a moderate stringency is 2 x SSC
25 buffer, 0.1% w/v SDS at a temperature in the range 20°C to 65°C; high stringency is 0.1 x SSC buffer, 0.1% w/v SDS at a temperature of at least 65°C.

The present invention is exemplified herein in relation to the defensin-like molecules from *N. alata*. However, this is done with the understanding that the present invention extends to
30 any novel floral-derived, defensin-like molecule from any plant provided the molecule has activity against plant pests and in particular insects. The present invention extends to

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derivatives of defensin-like molecules including heterologous molecules as well as the use of known defensins as anti-insect molecules.

Reference to a "defensin-like molecule" is made to highlight the fact that the present invention extends to homologues of defensin molecules.

The present invention further provides genetic constructs for use in expressing defensin-like molecule-encoding nucleotide sequences in plants for the purposes of protecting the plant from plant pests.

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Accordingly, another aspect of the present invention provides a genetic construct comprising a promoter or functional equivalent thereof operably linked to a nucleotide sequence encoding a floral-derived, defensin-like molecule having a mature domain comprising the amino acid sequence:-

15

a₁a₂C_Ia₃a₄a₅a₆a₇a₈a₉a₁₀a₁₁a₁₂C_{II}a₁₃a₁₄a₁₅a₁₆a₁₇C_{III}a₁₈a₁₉a₂₀C_{IV}a₂₁a₂₂a₂₃a₂₄a₂₅
a₂₆a₂₇a₂₈a₂₉C_Va₃₀a₃₁a₃₂a₃₃a₃₄a₃₅C_{VI}a₃₆C_{VII}a₃₇a₃₈a₃₉C_{VIII}

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wherein "a" may be the same or different and represents any amino acid residue, the numerical subscript on each "a" represents its position in the amino acid sequence and "C" represents a cysteine residue at a position indicated by its Roman numeral and wherein said mature domain exhibits inhibitory activity against plant pests such as insect pests with the proviso that the defensin-like molecule is not FST or TPP3.

25 In a preferred embodiment, the amino acid sequence of the mature domain comprises the amino acid sequence [SEQ ID NO:58]:-

X₃₀X₃₁CX₃₂X₃₃X₃₄SX₃₅X₃₆FX₃₇GX₃₈CX₃₉X₄₀X₄₁X₄₂X₄₃CX₄₄X₄₅X₄₆CX₄₇X₄₈EX₄₉
FX₅₀X₅₁GX₅₂CX₅₃X₅₄X₅₅X₅₆X₅₇X₅₈CX₅₉CTX₆₀X₆₁C

30

wherein X₃₀ = R or Q

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	X ₃₁	=	E, I or T
	X ₃₂	=	K or E
	X ₃₃	=	T, A or S
	X ₃₄	=	E, P or Q
5	X ₃₅	=	N, Q or H
	X ₃₆	=	T or R
	X ₃₇	=	P, K or H
	X ₃₈	=	I, L, P or T
	X ₃₉	=	I, F, S or V
10	X ₄₀	=	T, M, R or S
	X ₄₁	=	K, D, E or A
	X ₄₂	=	P or S
	X ₄₃	=	P, S or N
	X ₄₄	=	R or A
15	X ₄₅	=	K, T, S or N
	X ₄₆	=	A, Y or V
	X ₄₇	=	I, L, Q or H
	X ₄₈	=	S, K, T or N
	X ₄₉	=	K or G
20	X ₅₀	=	T, S, I, or V
	X ₅₁	=	D or G
	X ₅₂	=	H, R, or N
	X ₅₃	=	S, P or R
	X ₅₄	=	K, W, A or G
25	X ₅₅	=	I, L or F
	X ₅₆	=	L, Q, P or R
	X ₅₇	=	R or P
	X ₅₈	=	R or K
	X ₅₉	=	L or F
30	X ₆₀	=	K, S or R
	X ₆₁	=	P, N or H

The mature domain-encoding sequence may be operably linked to a signal domain comprising the amino acid sequence [SEQ ID NO:59]:-

$$5 \quad \text{MX}_1\text{X}_2\text{SX}_3\text{X}_4\text{X}_5\text{X}_6\text{X}_7\text{X}_8\text{X}_9\text{X}_{10}\text{X}_{11}\text{X}_{12}\text{X}_{13}\text{X}_{14}\text{X}_{15}\text{X}_{16}\text{X}_{17}\text{X}_{18}\text{X}_{19}\text{X}_{20}\text{X}_{21}\text{X}_{22} \\ \text{X}_{23}\text{X}_{24}\text{X}_{25}\text{X}_{26}\text{X}_{27}\text{X}_{28}\text{AX}_{29}$$

	wherein	X ₁	=	A, G or K
		X ₂	=	R, N, or L
10		X ₃	=	L, I or M
		X ₄	=	C, F or R
		X ₅	=	F or L
		X ₆	=	M, F or I
		X ₇	=	A or S
15		X ₈	=	F, T or A
		X ₉	=	A, L, V or F
		X ₁₀	=	I, V, L or F
		X ₁₁	=	L or I
		X ₁₂	=	A, I or M
20		X ₁₃	=	M, A or F
		X ₁₄	=	M or L
		X ₁₅	=	L or I
		X ₁₆	=	F or V
		X ₁₇	=	V, T or L
25		X ₁₈	=	A, T or S
		X ₁₉	=	Y or T
		X ₂₀	=	E or G
		X ₂₁	=	V or M
		X ₂₂	=	no amino acid or G
30		X ₂₃	=	no amino acid or P
		X ₂₄	=	no amino acid, M or V

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C-terminal domain comprising the sequence [SEQ ID NO:60]:-

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X ₈₁	=	no amino acid or T
X ₈₂	=	no amino acid or L
X ₈₃	=	no amino acid or A or S
X ₈₄	=	no amino acid or A or E
X ₈₅	=	no amino acid or A or V
X ₈₆	=	no amino acid or L or V
X ₈₇	=	no amino acid or L
X ₈₈	=	no amino acid or E
X ₈₉	=	no amino acid or E
X ₉₀	=	no amino acid or E
X ₉₁	=	no amino acid or I
X ₉₂	=	no amino acid or M
X ₉₃	=	no amino acid or D or M
X ₉₄	=	no amino acid or N or E

In yet another embodiment, the defensin-like molecule comprises the sequence [SEQ ID NO:61]:-

20

MX₁X₂SX₃X₄X₅X₆X₇X₈X₉X₁₀X₁₁X₁₂X₁₃X₁₄X₁₅X₁₆X₁₇X₁₈X₁₉X₂₀X₂₁X₂₂X₂₃X₂₄X₂₅
X₂₆X₂₇X₂₈AX₂₉X₃₀X₃₁CX₃₂X₃₃X₃₄SX₃₅X₃₆FX₃₇GX₃₈CX₃₉X₄₀X₄₁X₄₂X₄₃CX₄₄X₄₅X₄₆C
X₄₇X₄₈EX₄₉FX₅₀X₅₁GX₅₂CX₅₃X₅₄X₅₅X₅₆X₅₇X₅₈X₅₉CTX₆₀X₆₁CX₆₂X₆₃X₆₄X₆₅
X₆₆X₆₇X₆₈X₆₉X₇₀X₇₁X₇₂X₇₃X₇₄X₇₅X₇₆X₇₇X₇₈X₇₉X₈₀X₈₁X₈₂X₈₃X₈₄X₈₅X₈₆X₈₇X₈₈X₈₉X₉₀
X₉₁X₉₂X₉₃X₉₄

25 wherein

X ₁	=	A, G or K
X ₂	=	R, N, or L
X ₃	=	L, I or M
X ₄	=	C, F or R
X ₅	=	F or L
30 X ₆	=	M, F or I
X ₇	=	A or S

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	X_8	=	F, T or A
	X_9	=	A, L, V or F
	X_{10}	=	I, V, L or F
	X_{11}	=	L or I
5	X_{12}	=	A, I or M
	X_{13}	=	M, A or F
	X_{14}	=	M or L
	X_{15}	=	L or I
	X_{16}	=	F or V
10	X_{17}	=	V, T or L
	X_{18}	=	A, T or S
	X_{19}	=	Y or T
	X_{20}	=	E or G
	X_{21}	=	V or M
15	X_{22}	=	no amino acid or G
	X_{23}	=	no amino acid or P
	X_{24}	=	no amino acid, M or V
	X_{25}	=	no amino acid or T
	X_{26}	=	no amino acid, I or S
20	X_{27}	=	no amino acid or A or V
	X_{28}	=	Q or E
	X_{29}	=	no amino acid or Q
	X_{30}	=	R or Q
	X_{31}	=	E, I or T
25	X_{32}	=	K or E
	X_{33}	=	T, A or S
	X_{34}	=	E, P or Q
	X_{35}	=	N, Q or H
	X_{36}	=	T or R
30	X_{37}	=	P, K or H
	X_{38}	=	I, L, P or T

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	X ₃₉	=	I, F, S or V
	X ₄₀	=	T, M, R or S
	X ₄₁	=	K, D, E or A
	X ₄₂	=	P or S
5	X ₄₃	=	P, S or N
	X ₄₄	=	R or A
	X ₄₅	=	K, T, S or N
	X ₄₆	=	A, Y or V
	X ₄₇	=	I, L, Q or H
10	X ₄₈	=	S, K, T or N
	X ₄₉	=	K or G
	X ₅₀	=	T, S, I, or V
	X ₅₁	=	D or G
	X ₅₂	=	H, R, or N
15	X ₅₃	=	S, P or R
	X ₅₄	=	K, W, A or G
	X ₅₅	=	I, L or F
	X ₅₆	=	L, Q, P or R
	X ₅₇	=	R or P
20	X ₅₈	=	R or K
	X ₅₉	=	L or F
	X ₆₀	=	K, S or R
	X ₆₁	=	P, N or H
	X ₆₂	=	no amino acid or V
25	X ₆₃	=	no amino acid or F
	X ₆₄	=	no amino acid or D
	X ₆₅	=	no amino acid or E or K
	X ₆₆	=	no amino acid or K or I
	X ₆₇	=	no amino acid or M or S
30	X ₆₈	=	no amino acid or T, I or S
	X ₆₉	=	no amino acid or K or E

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	X ₇₀	=	no amino acid or T or V
	X ₇₁	=	no amino acid or G or K
	X ₇₂	=	no amino acid or A
	X ₇₃	=	no amino acid or E
5	X ₇₄	=	no amino acid or I or T
	X ₇₅	=	no amino acid or L
	X ₇₆	=	no amino acid or A, V or G
	X ₇₇	=	no amino acid or E
	X ₇₈	=	no amino acid or E
10	X ₇₉	=	no amino acid or A
	X ₈₀	=	no amino acid or K
	X ₈₁	=	no amino acid or T
	X ₈₂	=	no amino acid or L
	X ₈₃	=	no amino acid or A or S
15	X ₈₄	=	no amino acid or A or E
	X ₈₅	=	no amino acid or A or V
	X ₈₆	=	no amino acid or L OR v
	X ₈₇	=	no amino acid or L
	X ₈₈	=	no amino acid or E
20	X ₈₉	=	no amino acid or E
	X ₉₀	=	no amino acid or E
	X ₉₁	=	no amino acid or I
	X ₉₂	=	no amino acid or M
	X ₉₃	=	no amino acid or D or M
25	X ₉₄	=	no amino acid or N or E

In a preferred embodiment, the genetic construct comprises a nucleotide sequence encoding an amino acid sequence selected from SEQ ID NO:8, SEQ ID NO:14, SEQ ID NO:16 and SEQ ID NO:18.

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In a particularly preferred embodiment, the genetic construct comprises a nucleotide

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sequence selected from SEQ ID NO:7, SEQ ID NO:13, SEQ ID NO:15 and SEQ ID NO:17 or a nucleotide sequence having at least 70% similarity to one or more of SEQ ID NO:8, SEQ ID NO:14, SEQ ID NO:16 and SEQ ID NO:18 or a nucleotide sequence capable of hybridizing to SEQ ID NO:7, SEQ ID NO:13, SEQ ID NO:15 and SEQ ID NO:17 or a complementary form thereof.

Most preferably, the amino acid sequence corresponds to the mature domain and comprises the amino acid sequence set forth in SEQ ID NO:8.

Reference to SEQ ID NO:8, SEQ ID NO:14, SEQ ID NO:16 and SEQ ID NO:18 includes reference to novel variants having at least about 70% similarity to any one of SEQ ID NO:8, SEQ ID NO:14, SEQ ID NO:16 and SEQ ID NO:18. These sequences may also be used to generate multimeric or heterologous molecules.

In accordance with this aspect of the present invention, the construct is for use in generating transgenic plants with increased or enhanced resistance to plant pests (e.g. insect), attack or infestation. Preferably, the construct is used solely for this purpose. The construct may, however, be used for generating recombinant defensin-like molecules in microorganisms such as bacteria. The present invention extends to generic constructs encoding any defensin or defensin-like molecule for use in combating insect infestation. For example, such constructs may be used to generate transgenic plants resistant to insects.

Accordingly, the present invention provides a genetic construct for use in generating insect-resistant transgenic plants, said transgenic plants producing a defensin or defensin-like molecule selected from SEQ ID NO:8, SEQ ID NO:14, SEQ ID NO:16 and SEQ ID NO:18 as well as SEQ ID NO:20 to SEQ ID NO:49 or an amino acid sequence having at least 70% similarity to any one of SEQ ID NO:8, SEQ ID NO:14, SEQ ID NO:16 and SEQ ID NO:18.

As stated above, a plant includes either a monocotyledonous plant or dicotyledonous plant. Particularly, useful plants are food crops such as wheat, rice, barley, soybean and

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	X_{32}	=	K or E
	X_{33}	=	T, A or S
	X_{34}	=	E, P or Q
	X_{35}	=	N, Q or H
5	X_{36}	=	T or R
	X_{37}	=	P, K or H
	X_{38}	=	I, L, P or T
	X_{39}	=	I, F, S or V
	X_{40}	=	T, M, R or S
10	X_{41}	=	K, D, E or A
	X_{42}	=	P or S
	X_{43}	=	P, S or N
	X_{44}	=	R or A
	X_{45}	=	K, T, S or N
15	X_{46}	=	A, Y or V
	X_{47}	=	I, L, Q or H
	X_{48}	=	S, K, T or N
	X_{49}	=	K or G
	X_{50}	=	T, S, I, or V
20	X_{51}	=	D or G
	X_{52}	=	H, R, or N
	X_{53}	=	S, P or R
	X_{54}	=	K, W, A or G
	X_{55}	=	I, L or F
25	X_{56}	=	L, Q, P or R
	X_{57}	=	R or P
	X_{58}	=	R or K
	X_{59}	=	L or F
	X_{60}	=	K, S or R
30	X_{61}	=	P, N or H

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More preferably, the mature domain is selected from the amino acid sequences set forth in SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6 and SEQ ID NO:8.

- 5 Most preferably, the mature domain comprises an amino acid sequence set forth in SEQ ID NO:8.

Sub
Act Yet another aspect of the present invention provides a method for generating a plant with increased or enhanced resistance to an insect, said method comprising introducing into the
10 genome of a plant cell or genome of a group of plant cells a genetic construct comprising a promoter or functional equivalent thereof operably linked to a nucleotide sequence encoding a defensin-like molecule having a mature domain comprising the amino acid sequence:-

15 a₁a₂C_Ia₃a₄a₅a₆a₇a₈a₉a₁₀a₁₁a₁₂C_{II}a₁₃a₁₄a₁₅a₁₆a₁₇C_{III}a₁₈a₁₉a₂₀C_{IV}a₂₁a₂₂a₂₃a₂₄a₂₅
a₂₆a₂₇a₂₈a₂₉C_Va₃₀a₃₁a₃₂a₃₃a₃₄a₃₅C_{VI}a₃₆C_{VII}a₃₇a₃₈a₃₉C_{VIII}

wherein "a" may be the same or different and represents any amino acid residue, the numerical subscript on each "a" represents its position in the amino acid sequence and "C"
20 represents a cysteine residue at a position indicated by its Roman numeral and wherein said mature domain exhibits inhibitory activity against plant pests such as insect pests and regenerating a plant from said cell or group of cells.

Preferred defensins are selected from SEQ ID NO:8, SEQ ID NO:18 and SEQ ID NO:20
25 to SEQ ID NO:49.

The mature domains referred to above include fragments and derivatives of these domains.

The term "fragment" as used herein means a portion or a part of the mature domain parent
30 which preferably retains the activity of the parent mature domain. The term "fragment" includes deletions, mutants and small peptides, for example, of at least 5, preferably at

least about 10 and more preferably at least about 20 contiguous amino acids, which comprise the above anti-plant pest activity. Peptides of this type may be obtained through the application of standard recombinant nucleic acid techniques or synthesized using conventional liquid or solid phase synthesis as described, for example, in Chapter 9
5 entitled "*Peptide Synthesis*" by Atherton and Shephard which is included in a publication entitled "*Synthetic Vaccines*" edited by Nicholson and published by Blackwell Scientific Publications. Alternatively, peptides can be produced by digestion of an amino acid sequence of the invention with proteinases such as endoLys-C, endoArg-C, endoGlu-C and Staphylococcus V8-protease. The digested fragments can be purified, for example, by high
10 performance liquid chromatographic (HPLC) techniques.

By "derivative" is meant a polypeptide that has been derived from the basic sequence by modification, for example, by conjugation or complexing with other chemical moieties or by post-translational modification techniques as would be understood in the art. The term
15 "derivative" also includes within its scope alterations that have been made to a parent sequence including additions or deletions that provide for functionally-equivalent molecules. Accordingly, the term "derivative" encompasses molecules that affect a plant's phenotype in the same way as does the parent amino acid sequence from which it was generated. Also encompassed are polypeptides in which one or more amino acids have
20 been replaced by different amino acids. It is well understood in the art that some amino acids may be changed to others with broadly similar properties without changing the nature of the activity of the polypeptide (conservative substitutions) as described hereinafter. These terms also encompass polypeptides in which one or more amino acids have been added or deleted or replaced with different amino acids.

25

The terms "protein", "polypeptide", "peptide" and "an amino acid sequence" are used interchangeably herein to refer to a polymer of amino acid residues and to variants and synthetic analogues thereof. Thus, these terms apply to amino acid polymers in which one or more amino acid residues is a synthetic non-naturally occurring amino acid, such as a
30 chemical analogue of a corresponding naturally occurring amino acid as well as to naturally occurring amino acid polymers.

- 50 -

The terms "variant" and "homologue" refer to nucleotide sequences displaying substantial sequence identity with reference nucleotide sequences or polynucleotides that hybridize with a reference sequence under stringency conditions that are herein defined. The terms
5 "nucleic acid molecule", "nucleotide sequence", "polynucleotide" and "nucleic acid molecule" may be used herein interchangeably and encompass polynucleotides in which one or more nucleotides have been added or deleted or replaced with different nucleotides. In this regard, it is well understood in the art that certain alterations inclusive of mutations, additions, deletions and substitutions can be made to a reference nucleotide sequence
10 whereby the altered polynucleotide retains the biological function or activity of the reference polynucleotide. Such variant polypeptide sequences may encode polypeptides comprising some differences in their amino acid composition but nevertheless encoding a protein having the same or similar activity. The resulting variant polypeptide sequences are encompassed herein. The term "variant" also includes naturally occurring nucleotide allelic
15 variants.

The term "expression" is used in its broadest sense and includes transient, semi-permanent and stable expression, as well as inducible, tissue-specific, constitutive and/or developmentally-regulated expression. Stable, tissue-specific expression is preferred.
20

To effect expression of the nucleotide sequence of the present invention, it may conveniently be incorporated into a chimeric genetic construct comprising *inter alia* one or more of the following: a promoter sequence, a 5' non-coding region, a *cis*-regulatory region such as a functional binding site for transcriptional regulatory protein or
25 translational regulatory protein, an upstream activator sequence, an enhancer element, a silencer element, a TATA box motif, a CCAAT box motif, an upstream open reading frame, transcriptional start site, translational start site, and/or nucleotide sequence which encodes a leader sequence, termination codon, translational stop site and a 3' non-translated region. Preferably, the chimeric genetic construct is designed for transformation
30 of plants as hereinafter described.

The term "5' non-coding region" is used herein in its broadest context to include all nucleotide sequences which are derived from the upstream region of an expressible gene, other than those sequences which encode amino acid residues which comprise the polypeptide product of said gene, wherein 5' non-coding region confers or activates or otherwise facilitates, at least in part, expression of the gene.

The term "gene" is used in its broadest context to include both a genomic DNA region corresponding to the gene as well as a cDNA sequence corresponding to exons or a recombinant molecule engineered to encode a functional form of a product.

10

As used herein, the term "*cis*-acting sequence" or "*cis*-regulatory region" or similar term shall be taken to mean any sequence of nucleotides which is derived from an expressible genetic sequence wherein the expression of the first genetic sequence is regulated, at least in part, by said sequence of nucleotides. Those skilled in the art will be aware that a *cis*-regulatory region may be capable of activating, silencing, enhancing, repressing or otherwise altering the level of expression and/or cell-type-specificity and/or developmental specificity of any structural gene sequence.

Reference herein to a "promoter" is to be taken in its broadest context and includes the transcriptional regulatory sequences of a classical genomic gene, including the TATA box which is required for accurate transcription initiation, with or without a CCAAT box sequence and additional regulatory elements (i.e. upstream activating sequences, enhancers and silencers) which alter gene expression in response to developmental and/or environmental stimuli, or in a tissue-specific or cell-type-specific manner. A promoter is usually, but not necessarily, positioned upstream or 5', of a structural gene, the expression of which it regulates. Furthermore, the regulatory elements comprising a promoter are usually positioned within 2 kb of the start site of transcription of the gene.

In the present context, the term "promoter" is also used to describe a synthetic or fusion molecule, or derivative which confers, activates or enhances expression of a structural gene or other nucleic acid molecule, in a plant cell. Preferred promoters according to the

invention may contain additional copies of one or more specific regulatory elements to further enhance expression in a cell, and/or to alter the timing of expression of a structural gene to which it is operably connected.

- 5 The term "operably connected" or "operably linked" in the present context means placing a structural gene under the regulatory control of a promoter, which then controls the transcription and optionally translation of the gene. In the construction of heterologous promoter/structural gene combinations, it is generally preferred to position the genetic sequence or promoter at a distance from the gene transcription start site that is
- 10 approximately the same as the distance between that genetic sequence or promoter and the gene it controls in its natural setting, i.e. the gene from which the genetic sequence or promoter is derived. As is known in the art, some variation in this distance can be accommodated without loss of function. Similarly, the preferred positioning of a regulatory sequence element with respect to a heterologous gene to be placed under its control is
- 15 defined by the positioning of the element in its natural setting, i.e. the genes from which it is derived.

Promoter sequences contemplated by the present invention may be native to the host plant to be transformed or may be derived from an alternative source, where the region is

20 functional in the host plant. Other sources include the *Agrobacterium* T-DNA genes, such as the promoters for the biosynthesis of nopaline, octapine, mannopine, or other opine promoters; promoters from plants, such as the ubiquitin promoter; tissue specific promoters (see, e.g. U.S. Patent No. 5,459,252 to Conkling *et al.*; WO 91/13992 to Advanced Technologies); promoters from viruses (including host specific viruses), or

25 partially or wholly synthetic promoters. Numerous promoters that are functional in mono- and dicotyledonous plants are well known in the art (see, for example, Greve, *J. Mol. Appl. Genet.* 1: 499-511, 1983; Salomon *et al.*, *EMBO J.* 3: 1984; Garfinkel *et al.*, *Cell* 27: 143-513, 1983; Barker *et al.*, *Plant Mol. Biol.* 2: 235-350, 1983) including various promoters isolated from plants (such as the Ubi promoter from the maize *ubi-1* gene) (see, e.g. U.S.

30 Patent No. 4,962,028) and viruses (such as the cauliflower mosaic virus promoter, CaMV 35S).

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The promoter sequences may include regions which regulate transcription, where the regulation involves, for example, chemical or physical repression or induction (e.g. regulation based on metabolites, light, or other physicochemical factors; see, e.g. WO 93/06710 disclosing a nematode responsive promoter) or regulation based on cell differentiation (such as associated with leaves, roots, seed, or the like in plants; see, e.g. U.S. Patent No. 5,459,252 disclosing a root-specific promoter). Thus, the promoter region, or the regulatory portion of such region, is obtained from an appropriate gene that is so regulated. For example, the 1,5-ribulose biphosphate carboxylase gene is light-induced and may be used for transcriptional initiation. Other genes are known which are induced by stress, temperature, wounding, pathogen effects, etc.

The chimeric genetic construct of the present invention may also comprise a 3' non-translated sequence. A 3' non-translated sequence refers to that portion of a gene comprising a DNA segment that contains a polyadenylation signal and any other regulatory signals capable of effecting mRNA processing or gene expression. The polyadenylation signal is characterized by effecting the addition of polyadenylic acid tracts to the 3' end of the mRNA precursor. Polyadenylation signals are commonly recognized by the presence of homology to the canonical form 5' AATAAA-3' although variations are not uncommon.

The 3' non-translated regulatory DNA sequence preferably includes from about 50 to 1,000 nucleotide base pairs and may contain plant transcriptional and translational termination sequences in addition to a polyadenylation signal and any other regulatory signals capable of effecting mRNA processing or gene expression. Examples of suitable 3' non-translated sequences are the 3' transcribed non-translated regions containing a polyadenylation signal from the nopaline synthase (*nos*) gene of *Agrobacterium tumefaciens* (Bevan *et al.*, *Nucl. Acid Res.* 11: 369, 1983) and the terminator for the T7 transcript from the octopine synthase gene of *Agrobacterium tumefaciens*. Alternatively, suitable 3' non-translated sequences may be derived from plant genes such as the 3' end of the protease inhibitor I or II genes from potato or tomato, the soybean storage protein

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genes and the pea E9 small subunit of the ribulose-1,5-bisphosphate carboxylase (ssRUBISCO) gene, although other 3' elements known to those of skill in the art can also be employed. Alternatively, 3' non-translated regulatory sequences can be obtained *de novo* as, for example, described by An (*Methods in Enzymology* 153: 292, 1987), which is
5 incorporated herein by reference.

A chimeric genetic construct can also be introduced into a vector, such as a plasmid. Plasmid vectors include additional DNA sequences that provide for easy selection, amplification and transformation of the expression cassette in prokaryotic and eukaryotic
10 cells, e.g. pUC-derived vectors, pSK-derived vectors, pGEM-derived vectors, pSP-derived vectors, or pBS-derived vectors. Additional DNA sequences include origins of replication to provide for autonomous replication of the vector, selectable marker genes, preferably encoding antibiotic or herbicide resistance, unique multiple cloning sites providing for multiple sites to insert DNA sequences or genes encoded in the chimeric genetic construct,
15 and sequences that enhance transformation of prokaryotic and eukaryotic cells.

The vector preferably contains an element(s) that permits either stable integration of the vector or a chimeric genetic construct contained therein into the host cell genome, or autonomous replication of the vector in the cell independent of the genome of the cell. The
20 vector, or a construct contained therein, may be integrated into the host cell genome when introduced into a host cell. For integration, the vector may rely on a foreign or endogenous DNA sequence present therein or any other element of the vector for stable integration of the vector into the genome by homologous recombination. Alternatively, the vector may contain additional nucleic acid sequences for directing integration by homologous
25 recombination into the genome of the host cell. The additional nucleic acid sequences enable the vector or a construct contained therein to be integrated into the host cell genome at a precise location in the chromosome. To increase the likelihood of integration at a precise location, the integrational elements should preferably contain a sufficient number of nucleic acids, such as 100 to 1,500 base pairs, preferably 400 to 1,500 base pairs, and
30 most preferably 800 to 1,500 base pairs, which are highly homologous with the corresponding target sequence to enhance the probability of homologous recombination.

The integrational elements may be any sequence that is homologous with the target sequence in the genome of the host cell. Furthermore, the integrational elements may be non-encoding or encoding nucleic acid sequences.

- 5 For cloning and sub-cloning purposes, the vector may further comprise an origin of replication enabling the vector to replicate autonomously in a host cell such as a bacterial cell. Examples of bacterial origins of replication are the origins of replication of plasmids pBR322, pUC19, pACYC177, and pACYC184 permitting replication in *E. coli*, and pUB110, pE194, pTA1060, and pAM β 1 permitting replication in *Bacillus*. The origin of
10 replication may be one having a mutation to make its function temperature-sensitive in a *Bacillus* cell (see, e.g. Ehrlich, *Proc. Natl. Acad. Sci. USA* 75: 1433, 1978).

- To facilitate identification of transformed cells, the vector desirably comprises a further genetic construct comprising a selectable or screenable marker gene. The actual choice of a
15 marker is not crucial as long as it is functional (i.e. selective) in combination with the plant cells of choice. The marker gene and the nucleotide sequence of interest do not have to be linked, since co-transformation of unlinked genes as, for example, described in U.S. Patent No. 4,399,216 is also an efficient process in plant transformation.

- 20 Included within the terms selectable or screenable marker genes are genes that encode a "secretable marker" whose secretion can be detected as a means of identifying or selecting for transformed cells. Examples include markers that encode a secretable antigen that can be identified by antibody interaction, or secretable enzymes that can be detected by their catalytic activity. Secretable proteins include, but are not restricted to, proteins that are
25 inserted or trapped in the cell wall (e.g. proteins that include a leader sequence such as that found in the expression unit of extensin or tobacco PR-S); small, diffusible proteins detectable, for example, by ELISA; and small active enzymes detectable in extracellular solution such as, for example, α -amylase, β -lactamase, phosphinothricin acetyltransferase).

- 30 Examples of bacterial selectable markers are the *dal* genes from *Bacillus subtilis* or *Bacillus licheniformis*, or markers that confer antibiotic resistance such as ampicillin,

kanamycin, erythromycin, chloramphenicol or tetracycline resistance. Exemplary selectable markers for selection of plant transformants include, but are not limited to, a *hyg* gene which encodes hygromycin B resistance; a neomycin phosphotransferase (*neo*) gene conferring resistance to kanamycin, paromomycin, G418 and the like as, for example, described by Potrykus *et al.* (*Mol. Gen. Genet.* 199: 183, 1985); a glutathione-S-transferase gene from rat liver conferring resistance to glutathione derived herbicides as, for example, described in EP-A 256 223; a glutamine synthetase gene conferring, upon overexpression, resistance to glutamine synthetase inhibitors such as phosphinothricin as, for example, described WO 87/05327, an acetyl transferase gene from *Streptomyces viridochromogenes* conferring resistance to the selective agent phosphinothricin as, for example, described in EP-A 275 957, a gene encoding a 5-enolshikimate-3-phosphate synthase (EPSPS) conferring tolerance to N-phosphonomethylglycine as, for example, described by Hinchee *et al.* (*Biotech.* 6: 915, 1988), a *bar* gene conferring resistance against bialaphos as, for example, described in WO 91/02071; a nitrilase gene such as *bxn* from *Klebsiella ozaenae* which confers resistance to bromoxynil (Stalker *et al.*, *Science* 242: 419, 1988); a dihydrofolate reductase (DHFR) gene conferring resistance to methotrexate (Thillet *et al.*, *J. Biol. Chem.* 263: 12500, 1988); a mutant acetolactate synthase gene (ALS), which confers resistance to imidazolinone, sulfonylurea or other ALS-inhibiting chemicals (EP-A-154 204); a mutated anthranilate synthase gene that confers resistance to 5-methyl tryptophan; or a dalapon dehalogenase gene that confers herbicide resistance.

Preferred screenable markers include, but are not limited to, a *uidA* gene encoding a β -glucuronidase (GUS) enzyme for which various chromogenic substrates are known; a β -galactosidase gene encoding an enzyme for which chromogenic substrates are known; an aequorin gene (Prasher *et al.*, *Biochem. Biophys. Res. Comm.* 126: 1259, 1985), which may be employed in calcium-sensitive bioluminescence detection; a green fluorescent protein gene (Niedz *et al.*, *Plant Cell Reports* 14: 403, 1995); a luciferase (*luc*) gene (Ow *et al.*, *Science* 234: 856, 1986), which allows for bioluminescence detection; a β -lactamase gene (Sutcliffe, *Proc. Natl. Acad. Sci. USA* 75: 3737, 1978), which encodes an enzyme for which various chromogenic substrates are known (e.g. PADAC, a chromogenic cephalosporin); an R-locus gene, encoding a product that regulates the production of

anthocyanin pigments (red colour) in plant tissues (Dellaporta *et al.*, *Chromosome Structure and Function*, pp. 263-282, 1988); an α -amylase gene (Ikuta *et al.*, *Biotech.* 8: 241, 1990); a tyrosinase gene (Katz *et al.*, *J. Gen. Microbiol.* 129: 2703, 1983) which encodes an enzyme capable of oxidizing tyrosine to dopa and dopaquinone which in turn
5 condenses to form the easily detectable compound melanin; or a *xylE* gene (Zukowsky *et al.*, *Proc. Natl. Acad. Sci. USA* 80: 1101, 1983), which encodes a catechol dioxygenase that can convert chromogenic catechols.

The selectable marker gene construct may also comprise any one or more of 5' and 3' non-
10 coding regions, *cis*-regulatory regions, enhancers, activators and the like, as hereinbefore described.

A further aspect of the present invention provides a transfected or transformed cell, tissue or organ from a plant or a transformed microbial cell, said cell, tissue or organ comprising
15 a nucleic acid molecule comprising a sequence of nucleotides encoding or complementary to a sequence encoding a polypeptide comprising, in its precursor form, an N-terminal signal domain, a mature domain and an acidic C-terminal domain wherein said polypeptide is produced during flower development and its mature domain has activity against one or more plant pests.

20

Sub 57
 25 Preferably, the nucleic acid molecule comprises a sequence of nucleotides encoding or complementary to a sequence encoding a polypeptide comprising, in its precursor form, an N-terminal signal domain, a mature domain and an acidic C-terminal domain wherein said polypeptide is produced during flower development and its mature domain comprises the structure:-

$a_1a_2C_Ia_3a_4a_5a_6a_7a_8a_9a_{10}a_{11}a_{12}C_{II}a_{13}a_{14}a_{15}a_{16}a_{17}C_{III}a_{18}a_{19}a_{20}C_{IV}a_{21}a_{22}a_{23}a_{24}a_{25}$
 $a_{26}a_{27}a_{28}a_{29}C_Va_{30}a_{31}a_{32}a_{33}a_{34}a_{35}C_{VI}a_{36}C_{VII}a_{37}a_{38}a_{39}C_{VIII}$

30 wherein "a" may be the same or different and represents any amino acid residue, the numerical subscript on each "a" represents its position in the amino acid sequence and "C"

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represents a cysteine residue at a position indicated by its Roman numeral and wherein the mature domain has activity against one or more plant pests.

The vectors and chimeric genetic construct(s) of the present invention may be introduced into a cell by various techniques known to those skilled in the art. The technique used may vary depending on the known successful techniques for that particular organism.

Techniques for introducing vectors, chimeric genetic constructs and the like into cells include, but are not limited to, transformation using CaCl_2 and variations thereof, direct DNA uptake into protoplasts, PEG-mediated uptake to protoplasts, microparticle bombardment, electroporation, microinjection of DNA, microparticle bombardment of tissue explants or cells, vacuum-infiltration of tissue with nucleic acid, and T-DNA-mediated transfer from *Agrobacterium* to the plant tissue.

For microparticle bombardment of cells, a microparticle is propelled into a cell to produce a transformed cell. Any suitable ballistic cell transformation methodology and apparatus can be used in performing the present invention. Exemplary procedures are disclosed in Sanford and Wolf (U.S. Patent Nos. 4,945,050, 5,036,006, 5,100,792, 5,371,015). When using ballistic transformation procedures, the genetic construct may incorporate a plasmid capable of replicating in the cell to be transformed.

Examples of microparticles suitable for use in such systems include 0.1 to 10 μm and more particularly 10.5 to 5 μm tungsten or gold spheres. The DNA construct may be deposited on the microparticle by any suitable technique, such as by precipitation.

Plant tissue capable of subsequent clonal propagation, whether by organogenesis or embryogenesis, may be transformed with a chimeric genetic construct of the present invention and a whole plant generated therefrom. The particular tissue chosen will vary depending on the clonal propagation systems available for, and best suited to, the particular species being transformed. Exemplary tissue targets include leaf disks, pollen, embryos, cotyledons, hypocotyls, megagametophytes, callus tissue, existing meristematic tissue (e.g.

apical meristem, axillary buds, and root meristems), and induced meristem tissue (e.g. cotyledon meristem and hypocotyl meristem).

5 The regenerated transformed plants may be propagated by a variety of means, such as by clonal propagation or classical breeding techniques. For example, a first generation (or T1) transformed plant may be selfed to give a homozygous second generation (or T2) transformant and the T2 plants further propagated through classical breeding techniques.

10 Accordingly, this aspect of the present invention, insofar as it relates to plants, further extends to progeny of the plants engineered to express the nucleic acid molecule encoding the defensin-like molecule or a variant or homologue thereof as well as vegetative, propagative and reproductive parts of the plants, such as flowers (including cut or severed flowers), parts of plants, fibrous material from plants (for example, cotton) and reproductive portions including cuttings, pollen, seeds and callus.

15 Another aspect of the present invention provides a genetically modified plant cell or multicellular plant or progeny thereof or parts of a genetically modified plant capable of producing a heterologous defensin-like molecule as herein described wherein said transgenic plant is resistant or has reduced sensitivity to plant pests such as insects.

20 More particularly, the present invention provides a genetically modified plant cell or multicellular plant or progeny or parts thereof comprising the amino acid sequence set forth in SEQ ID NO:8 or a fragment or derivative.

25 The term "genetically modified" is used in its broadest sense and includes introducing gene(s) into cells, mutating gene(s) in cells and altering or modulating the regulation of gene(s) in cells.

30 The genetic construct may be a single molecule or multiple molecules such as a set of molecules such that a combination may comprise nucleotide sequences capable of encoding other anti-plant pathogen molecules such as but not limited to a proteinase

inhibitor or precursor thereof. Proteinase inhibitors such as serine proteinase inhibitors frequently accumulate in storage organs and in leaves in response to wounding. The inhibitory activities of the proteins are directed against a wide range of proteinases of microbial and animal origin.

5

Accordingly, another aspect of the present invention comprises one or more genetic constructs alone or in combination comprising a first promoter operably linked to a first nucleotide sequence wherein said first nucleotide sequence encodes a defensin-like molecule or part thereof capable of inhibiting a plant pest such as an insect, said construct
10 further comprising a second promoter operably linked to a second nucleotide sequence wherein said second nucleotide sequence encodes a proteinase inhibitor or precursor thereof.

In one embodiment, the defensin-like molecule and/or its encoding DNA sequence is
15 selected from SEQ ID NO:7 to SEQ ID NO:18. In another embodiment, the defensin-like molecule and/or its encoding DNA sequence is selected from SEQ ID NO:7 to SEQ ID NO:18 or SEQ ID NO:20 to SEQ ID NO:24. The defensin-like molecule and/or its encoding DNA sequence defined by SEQ ID NO:7 or SEQ ID NO:8 is particularly preferred. In yet another embodiment, the defensin-like molecule and/or its encoding DNA
20 sequence is selected from SEQ ID NO:7 or SEQ ID NO:8, SEQ ID NO:17 or SEQ ID NO:18 or SEQ ID NO:20 to SEQ ID NO:55.

In another embodiment, the proteinase inhibitor is a serine proteinase inhibitor of type I or type II. A particularly useful proteinase inhibitor comprises the amino acid sequence
25 encoding the nucleotide sequence set forth in SEQ ID NO:57 and SEQ ID NO:56, respectively. This molecule is also described in International Patent Application No. PCT/AU93/00659 (WO 94/13810).

The present invention extends to plants and plant parts including reproductive parts of
30 plants which carry all or part of a genetic construct which confers on the plant or plant parts resistance or reduced sensitivity to plant pests including insects and optionally a

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second genetic construct as described above.

The present invention further contemplates a composition comprising a defensin or defensin-like molecule alone or in combination with another agent such as a proteinase inhibitor. The composition is particularly useful for topical application to plants such as cotton plants, to assist in controlling insect infestation.

The present invention further contemplates a promoter associated with the genomic form of the nucleotide sequence set forth in SEQ ID NO:7. According to this aspect of the present invention, there is provided an isolated nucleic acid molecule having promoter activity and corresponding to the genomic region in a plant genome which is operably linked to a nucleotide sequence corresponding to all or part of SEQ ID NO:7 or a nucleotide sequence having 70% similarity thereof or a nucleotide sequence capable of hybridizing to SEQ ID NO:7 or its complementary form.

The present invention is further described by the following non-limiting Examples.

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EXAMPLE 1

Preparation of plant material

Nicotiana alata (Link et Otto) plants of mixed self-incompatibility genotype were maintained under glasshouse conditions as described by Anderson *et al.* (*Plant Cell* 1: 483-491, 1989). Flowers and floral buds were harvested and within two hours, pistils, ovaries and anthers were removed with forceps and a scalpel blade, while petals were separated by hand. Pollen grains from dehisced anthers and whole flowers at various stages of development were also collected. The tissues were frozen in liquid nitrogen and stored at -70°C until use.

EXAMPLE 2

Cloning of cDNA from N. alata

(a) *Isolation of RNA*

Total RNA was prepared by grinding 70 pistils (1.3 g) from *N. alata* flowers at the petal coloration stage of development to a fine powder with a sterile mortar and pestle in the presence of liquid nitrogen. The RNA was extracted with TRIzol (trademark) Reagent (Gibco BRL) according to the manufacturer's instructions (see Gibco BRL form #3796, TRIzol (trademark) Reagent Total RNA isolation reagent).

(b) *cDNA synthesis and amplification of floral defensin sequence by PCR*

First strand cDNA was prepared from total pistil RNA using the Superscript Preamplification System (Gibco BRL). Oligonucleotide primers used for PCR were specific to the DNA sequence published for the Flower Specific Thionin (FST, Gu *et al.* [1992; *supra*] from *N. tabacum*.

Primer FST1: 5' GGAATTCCATATGGCTCGCTCCTTGTGC 3' [SEQ ID NO:1]

Primer FST2 5' GCGGATCCTCAGTTATCCATTATCTCTTC 3' [SEQ ID NO:2]

Primer FST1 and primer FST2 matched the sequence of FST between nucleotides 49-66, and 346-363 respectively. A cDNA clone encoding the *N. alata* floral defensin precursor (*NaPdfl*) was obtained by PCR amplification using the single-stranded cDNA as a template. The *NaPdfl* product was cloned into the pBluescript SK+ II (Stratagene) vector (pBS-*NaPdfl*) for sequencing. The product is 318 bp in length and encodes the complete coding sequence without the 5' and 3' untranslated regions.

The *NaPdfl* PCR product was subsequently used to screen a previously constructed *N. alata* pistil cDNA library (Schultz *et al.*, *Plant Mol Biol* 35: 833-845, 1997). The membranes were probed with *NaPdfl* cDNA labelled with [α^{32} P]dCTP using random nonamer priming (Megaprime (trademark) DNA labelling kit, Amersham Life Technologies). Unincorporated [α^{32} P]dCTP was removed using a Bio-Rad Bio-Spin column 30 as described in the manufacturer's instructions. The blot was hybridized with the probe in a solution of 50% v/v formamide, 5 x SSPE, 5 x Denhardt's solution and 200 μ g/ml herring sperm DNA at 42°C for 16 h before unbound probe was removed by washing twice with 2 x SSPE and 0.1% w/v SDS at room temperature for 10 min. Hybridized probe was visualized by exposing the blot to x-ray film. Positive clones from the screen were excised and sequenced.

EXAMPLE 3

NaPdfl gene expression

(a) RNA gel blot analysis

Total RNA was isolated from anthers at stages I (5-10 mm buds), II (20-30 mm buds) and III (50-70 mm buds) of development, from pollen grains and from mature pistil, ovary, petal, leaf and root tissues of *N. alata* (self-incompatibility genotype, S_2S_2). The RNA samples (12.5 μ g) were fractionated on a denaturing 1% w/v agarose-formaldehyde gel and transferred to Hybond-N (Amersham Life Sciences) membrane. Production of a radio-labelled *NaPdfl* cDNA probe and hybridization conditions were as described for the

screening of the cDNA library, in Example 2(b), above. Stringency washes were in 0.2 x SSPE, 0.1% SDS at 45°C and 55°C for 30 min, respectively. Results are shown in Figure 2. Hybridized probe was visualized by exposing the blot to a storage phosphor screen for 24 h. The results were read in a Molecular Dynamics 400B phosphorimager, using the ImageQuant software.

(b) *In situ hybridization*

In situ hybridization was performed essentially as described by Drews *et al.* (*Cell* 65: 991-1002, 1991) and Cox and Goldberg (In: *Analysis of plant genes expression In Plant Molecular Biology: A Practical Approach*. C. H. Shaw (ed), pp. 1-34, IRL Press, Oxford, United Kingdom, 1988). ³⁵S-labelled sense and antisense RNA probes were produced by linearising the pBS-NaPdf1 DNA with *Eco*RI and *Bam*HI and transcribing with T7 and T3 RNA polymerases respectively (Ausubel *et al.* [1994; *supra*]; Drews *et al.* [1991; *supra*])

Ten mm flower buds were excised from the plant and fixed in 50% v/v ethanol, 5% v/v acetic acid and 3.7% v/v formaldehyde. The fixed tissues were dehydrated and embedded in paraffin (Sigma). The tissues were then sliced into 10 µm sections and attached to Superfrost⁺/plus slides (Menzel-Glaser, Germany). The sections were treated with xylene followed by hydration, proteinase K treatment, acetylation and dehydration. The ³⁵S-labelled sense and antisense riboprobes were hydrolyzed to about 100 nucleotides in length and hybridized to the sections at 42°C for 17 h. The sections were then treated with ribonuclease A and washed, before the slides were coated with film emulsion. The sections were developed after a 1 week exposure.

Results indicated that *NaPdf1* transcript accumulated in the connective tissue of the anthers, the cortical cells of the style and in the epidermal cells of the petals and sepals (refer to Figure 3). This accumulation pattern is consistent with the encoded protein playing a role in the defense of the reproductive organs.

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EXAMPLE 4***Protein expression for antibody production******(a) Molecular cloning and bacterial protein expression***

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The pBS-NaPdf1 (see Example 2(b)) was used to PCR amplify a DNA fragment encoding the proprotein domains (Napropdf1, precursor minus the N-terminal ER signal domain). For a schematic representation of the structure, see Figure 8. The Napropdf1 DNA fragment was obtained using oligonucleotide primers PDF1 and PDF2 which incorporated
10 a *Bam*HI and *Sac*I restriction site for subsequent cloning, respectively.

Primer PDF1 5' CCGGATCCAGAGAATGCAAAACAG 3' [SEQ ID NO:3]

Primer PDF2 5' GGGAGCTCTTAGTTATCCATTATCTC 3' [SEQ ID NO:4]

15 The Napropdf1 DNA fragment was cloned directly from PCR into the pGEM-T vector (Promega) according to the manufacturer's instructions and subcloned into the pQE30 (Qiagen) vector for protein expression in *E. coli* strain M15 bacteria (Qiagen).

The Napropdf1 protein was expressed in bacteria, as a fusion with an N-terminal
20 hexahistidine tag. Transformed *E. coli* cells were grown in LB broth containing ampicillin (100 µg/ml) and kanamycin (12.5 µg/ml) to an absorbance reading of ~0.8 at 595nm before induction with isopropyl β-D-thiogalactopyranoside (IPTG, 1 mM) for 6 h. Cells were pelleted by centrifugation and resuspended in lysis buffer (10 mM Tris-HCl pH 8.0, 100 mM sodium phosphate buffer pH 8.0, 8 M urea; 30 ml of lysis buffer/litre of culture)
25 before incubation for 30 min on ice. The lysate was then passed through an 18-gauge needle before the supernatant was collected by centrifugation (25,000 g, 15 min, 4°C). The hexahistidine-tagged proteins (6H.Napropdf1) were purified from lysed bacterial cells using the denaturing protein purification protocol outlined in the Clontech TALON (trademark) Metal Affinity Resin User Manual (PT1320-1). Bound proteins were eluted
30 from the resin in 100 mM EDTA, pH 8.0. The eluted proteins were lyophilized. Protein extracts corresponding to various steps in the purification procedure were analyzed by

SDS-PAGE (15% w/v polyacrylamide) and visualized by staining with Coomassie Blue (see Figure 4A). A sample of the metal affinity purified 6H.NaproPdf1 protein was applied to an Aquapore RP300 reverse-phase C8 analytical column (4.6 x 100 mm, Brownlee) using a Waters model 510 pump and a Waters model 481UV detector. Protein was eluted with a gradient of 0-100% buffer B (60% v/v acetonitrile in 0.089% v/v trifluoroacetic acid), over 30 min. The protein eluted in a single peak (Figure 4B) and N-terminal sequence and mass spectrometry confirmed that it was 6H.NaproPdf1.

(b) *Production of a polyclonal antiserum*

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The bacterially-expressed 6H.NaproPdf1 protein (1.3 mg) was conjugated to keyhole limpet hemocyanin (KLH, 0.3 mg, Sigma) with glutaraldehyde as described by Harlow and Lane (*Antibodies: A Laboratory Manual*. Cold Springs Harbor Laboratory Press, Cold Springs Harbor, New York, 1988), prior to injection into a rabbit. The conjugated protein was then dialyzed against PBS overnight. The protein conjugate (100 µg, in 1 mL PBS) was mixed with an equal volume of Freund's complete adjuvant (Sigma). The primary immunization consisted of 4 x 400 µL subcutaneous injections. Booster immunizations were administered 5 and 9 weeks later and contained the protein conjugate (100 µg, in 1 mL PBS) mixed with Freund's incomplete adjuvant (Sigma). Pre-immune serum was collected prior to injection while immune serum was collected 9 days after the second immunization.

(c) *Protein A purification of IgGs from whole sera*

25 The IgG fraction in the pre-immune serum and immune serum were purified using a Protein-A Sepharose CL-4B column (2.5 mL, Pharmacia) according to the manufacturer's instructions. The pre-immune serum (2 mL) was diluted in an equal volume of 0.1 M Tris-HCl (pH 7.5) and loaded onto the column which had been equilibrated in 0.1 M Tris-HCl (pH 7.5). The eluent was collected and re-applied three times. The column was washed with 80 mL of 0.1 M Tris-HCl (pH 7.5), followed by 80 mL of 0.01 M Tris-HCl (pH 7.5) to remove any unbound material. The IgGs were eluted with 100 mM glycine (pH 2.5) and

500 μ L fractions were collected into microfuge tubes containing 50 μ L of 1 M Tris-HCl (pH 8.8). Fractions containing the IgGs were pooled and dialysed extensively with PBS at 4°C. The Protein-A Sepharose column was regenerated by washing with 0.1 M Tris-HCl (pH 7.5). When the pH reached 7.5, the immune serum (3 mL, second bleed) was applied and purified as described for the pre-immune serum.

(d) *SDS-polyacrylamide gel electrophoresis*

Buffer (50 mM Tris-HCl pH 8.0, 10 mM EDTA, 0.5 M NaCl) soluble protein samples (60 μ g), from the stages of *N. alata* flower development shown in Figure 5(A), were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE, with 4% w/v stacking and 15% resolving w/v polyacrylamide gels) (Laemmli, *Nature* 227: 680-685, 1970) using a Mini Protean II Electrophoresis apparatus (Bio-Rad). The proteins were visualized by coomassie blue staining and compared to Broad Range molecular weight markers (6.5-200 kDa) from Bio-Rad.

(e) *Immunoblot analysis*

Proteins separated by SDS-PAGE were transferred to a nitrocellulose membrane (Micron Separations Inc., 0.22 μ m pore size) using a Mini-Protean II Trans-Blot apparatus (Bio-Rad) in transfer buffer (48 mM Tris (hydroxymethyl) aminomethane, 192 mM glycine, 20% v/v methanol). After transfer at 100 V for 1 h, the membrane was fixed in isopropanol for 1 min followed by a 5 min wash in TBS (20 mM Tris-HCl, 150 mM NaCl, pH 8.0). Molecular weight markers were visualized by amido black staining (1:50 dilution of 0.1% w/v amido black in 40% v/v methanol and 10% v/v acetic acid).

The membrane was blocked for 1 h in 5% w/v skim milk followed by incubation with anti-6H.Napropdf1 antibodies for 1.5 h (1:2500 dilution in TBS). The membrane was washed 3 x 10 min in TBST (0.1% v/v Tween 20 in TBS) before incubation with donkey anti-rabbit IgG conjugated to horseradish peroxidase for 1.5 h (Amersham Pharmacia Biotech; 1:5000 dilution in TBS). Three further 10 min washes were performed before the immunoblot was

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5 treated with Enhanced Chemi-Luminescence (ECL) detection reagents (Amersham Pharmacia Biotech) according to the manufacturer's instructions. Immunoblots were exposed to ECL Hyperfilm (Amersham Pharmacia Biotech). Three proteins reacted specifically with the antibodies and the levels of protein were most abundant during the early stages of development (refer to Figure 5B).

The lower molecular weight species was confirmed as the mature defensin by mass spectrometry (5.3 kDa) and N-terminal amino acid sequencing; refer to Example 5(c), below.

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EXAMPLE 5

Purification from floral buds

(a) *Protein extraction*

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Mature NaPdf1 protein was extracted from flowers using a modification of a procedure for extraction of thionins from barley flour (Ozaki *et al.*, *J. Biochem.* 87: 549-555, 1980). Whole *N. alata* flowers up to the petal coloration stage of flower development (5-50 mm, 650 g wet weight) were ground to a fine powder with liquid nitrogen using a mortar and pestle and processed further in an Ultra-Turrax homogenizer (Janke & Kunkel, Germany) in 50 mM sulfuric acid (3 mL/g weight). After stirring for 1 h at 4°C, insoluble material was removed by filtration through Miracloth (Calbiochem) followed by centrifugation (25,000 x g, 15 min, 4°C). The slurry was adjusted to pH 7.8 by the slow addition of 10 M NaOH and stirred for 1 h at 4°C before removal of precipitated material by centrifugation (25,000 x g, 15 min, 4°C). Solid ammonium sulfate was added to 80% (w/v) saturation and the mixture was stirred for 4 h or overnight at 4°C to precipitate the defensin protein. The precipitate was collected by centrifugation and was dissolved in 50 mL of gel filtration buffer (150 mM KCl, 10 mM Tris-HCl, pH 8.0) prior to heating at 90°C for 10 min. Following centrifugation, the supernatant was loaded onto a Sephadex G-50 gel filtration column (85 x 2.54 cm, Pharmacia). Fractions (50 mL) were collected and analyzed by immunoblotting with anti-6H.NaproPdf1 antibodies. Fractions containing

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NaPdl were pooled, concentrated by rotary evaporation at 45°C and filtered through a 0.22 µm syringe filter (Millipore) before further purification by RP-HPLC.

(b) *Reverse-phase high performance liquid chromatography*

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RP-HPLC was performed on a Beckman System Gold HPLC coupled to a Beckmann 166 detector. Analytical RP-HPLC was conducted on an Aquapore RP300 reverse-phase C8 column (4.6 x 100 mm, Brownlee) while preparative runs were performed using a Vydac C8 reverse-phase column (22 x 250 mm). The protein was eluted with a linear gradient of
10 0-100% buffer B (60% v/v acetonitrile in 0.089% v/v trifluoroacetic acid) at a flow-rate of 1 mL/min or 10 mL/min over 40 min, respectively. Results from the analytical column are shown in Figure 6A. The identity of the major peak was confirmed by N-terminal sequencing and mass spectrometry, as described below.

15 (c) *Electrospray ionisation mass spectrometry*

Prior to electrospray ionization mass spectrometry (ESI-MS), protein fractions from RP-HPLC were concentrated under vacuum in a freeze drier and reconstituted in milli-Q water. ESI-MS was carried out using 1-100 pmol protein in 2-4 µL of 50% v/v acetonitrile
20 containing 0.1% w/v formic acid. Samples were infused at a flow rate of 0.2 µL/min into a Perkin-Elmer Sciex API-300 triple quadrupole fitted with a micro-ionspray ion source. The mass scale was calibrated using singly-charged poly(propylene glycol) ions to a mass accuracy equivalent to ±1%. Mass spectra were recorded in the first quadrupole (Q1) scan mode over the mass range m/z 200 to 3000 daltons per charge using a constant peak width
25 (full width at half peak maximum) of 0.6 daltons per unit charge. Perkin-Elmer Sciex Bio-Multiview software was employed for signal averaging of 30-100 scans, manual mass determination and transformation of mass-to-charge ratio spectra to a true mass scale. Uncertainties were calculated at 95% confidence limits using small sample statistics and include calibration uncertainty.

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Results indicated that the protein in Peak A in Figure 6A, and the lower molecular weight species shown in Figure 5B (~5 kDa), corresponded to the mature defensin domain encoded by the *NaPdf1* cDNA clone (see Figure 6B).

5 (d) *Amino acid sequencing*

Amino acid sequencing by Edman degradation was carried out on an Applied Biosystems 470A gas-phase peptide sequenator coupled to an Applied Biosystems 130A separation system for automatic on-line PTH amino acid analysis. The eight amino acids of N-
10 terminal sequence obtained from the protein in Peak A matched the sequence predicted from the *NaPdf1* cDNA clone (see Figure 6B).

EXAMPLE 6

Immunogold localization

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Fixation and immunogold labelling for electron microscopy

Anthers and ovaries were removed from *N. alata* flowers at the immature bud stage (10 mm) and were fixed for 2 h at room temperature and then overnight at 4°C in 4% (w/v) formaldehyde and 0.5% (w/v) glutaraldehyde in 60 mM PIPES/KOH, pH 7.2. After
20 fixation, the tissues were washed in 60 mM PIPES/KOH, pH 7.2 and dehydrated for 3 h at room temperature in acidified dimethoxypropane (concentrated hydrochloric acid:dimethoxypropane, 1:2000 [v/v]). The dehydrated segments were embedded in LR Gold containing Benzil (London Resin Co. Ltd.) by polymerization under a Phillips TUV
25 15-W UV lamp at a distance of 10 cm for 12 h at 25°C. Immunogold labelling of ultrathin sections was performed as described in Anderson *et al.* (*Planta* 171: 438-442, 1987). The protein A purified anti-6H.NaproPdf1 antibodies were incubated with anther and ovary sections at a final concentration of 64 µg IgG/mL and 21 µg IgG/mL, respectively. Specificity of labeling was tested by replacing the primary antibody with antibodies
30 purified from pre-immune serum at the same concentration. For visualization of ultrastructure, the sections were stained for 15 min in 3% (w/v) aqueous uranyl acetate and

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2 min with Sato triple lead stain (Sato, *J. Electron Microsc.* 17: 158-159, 1968) before being viewed on a Joel 1200 electron microscope.

EXAMPLE 7

5 *Sequences and sequence comparisons*

The nucleotide and predicted amino acid sequences of *N. alata* defensin are set forth in Figure 1. The gene that the cDNA clone represents is designated *NaPdf1* (*N. alata* plant defensin 1). The DNA sequence shown is a composite sequence from an overlapping
 10 cDNA clone and a primer extension product as follows: 1-49, primer extension product clone; 50-541, cDNA clone *NaPdf1*. The protein predicted by this composite cDNA sequence is shown below the nucleotide sequence. The cDNA clone contains a single open reading frame of 318 nucleotides, encoding for 105 amino acids. The PCR amplified sequence in the original pBS-*NaPdf1* clone (see Example 2) corresponds to nucleotides 1-
 15 318 in the *NaPdf1* clone shown in Figure 1.

In Figure 9, the 105-amino acid sequence of *NaPdf1* (SEQ ID NO:18) is shown aligned with the predicted amino acid sequences encoded by five other flower-derived cDNA clones. The sequences, in order, are as follows: flower specific thionin (FST), sourced
 20 from Gu *et al.* [1992; *supra*] (SEQ ID NO: 20), derived from tobacco flowers; TPP3, sourced from Milligan and Gasser [1995; *supra*] (SEQ ID NO: 21), derived from tomato pistil; NTS13, sourced from Li and Gray [1999; *supra*] (SEQ ID NO: 22), derived from tobacco styles; PPT, sourced from Karunanandaa *et al.* [1994; *supra*] (SEQ ID NO: 23), derived from petunia pistil, and ATPIIIa, sourced from Yu *et al.* [1999; *supra*], Direct
 25 Submission, Accession No. S30578 (SEQ ID NO: 24), derived from *Arabidopsis*.

The 47 amino acids constituting the mature central domain of the *NaPdf1* protein (SEQ ID NO:8) were also aligned with the corresponding amino acid sequences of the mature domains of other members of the plant defensin family (SEQ ID NO:25 to SEQ ID
 30 NO:49). Alignment was carried out using the computerized algorithm of ClustalW (http://pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_clustalw.html). The results

are set forth in Figure 10. For details of the relevant references from which each sequence was obtained, and for their individual sequence identifiers, refer to the figure legend.

EXAMPLE 8

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Fungal growth inhibition assays

The 96-well microtitre plate assay of Broekaert *et al.* (*EMS Microbiology Letters* 69: 55-60, 1990) was used to test the effect of the purified NaPdf1 protein on the growth of *Botrytis cinerea* (isolated from rosemary, Brunswick, Victoria), *Fusarium oxysporum* (*f.* 10 *sp. dianthi*, Race 2; isolated from carnation by Florigene Limited, Collingwood, Victoria) and *Fusarium oxysporum* (*f. sp. vasinfectum*, isolate VCG 01111 from cotton; provided by the Department of Primary Industries, Queensland). Fungal spores were isolated from sporulating cultures growing on half strength potato dextrose agar (PDA, Difco) or gamma-irradiated carnation leaves in water agar by the addition of sterile water and the 15 use of a spreader. The suspension was filtered through two layers of autoclaved muslin and the spore concentration in the filtrate determined using a haemocytometer. The spores were used directly or after storage in sterile 20% v/v glycerol solution at -20°C.

Antifungal assays were performed in 96-well flat-bottomed microtitre plates (Greiner) 20 under aseptic conditions. The spore concentration was adjusted to about 2×10^4 spores/mL in PDB and 80 μ L of this suspension was added to each well to which 20 μ L of filter sterilised (0.22 μ m syringe filter, Millipore) test protein (10, 50 or 100 μ g/mL) or water was added. The purity and concentration of each protein was confirmed before use by RP-HPLC analysis. Sterile water and ovalbumin (Sigma) were added to other wells as negative 25 controls, and a mixture of the antifungal proteins α - and β -purothionin (Sigma) was used as a positive control. The plates were shaken on an orbital shaker for a few seconds to mix the spores and the test proteins. The plates were allowed to stand for 30 min to allow the spores to sediment before the optical density of the plates were determined using a Spectra Max Pro 250 microplate reader (Molecular Devices) at 595 nm absorbance. The plates 30 were incubated in the dark at 22°C and measurements were taken over the course of 100 h. An increase in absorbance relative to the initial reading was correlated with the growth of

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fungus hyphae and this was plotted against time. All assays were performed in quadruplicate.

Growth inhibition curves, set out in Figures 11 and 12, show the effect of purified NaPdlf defensin protein against *B. cinerea* (Figure 11), *F. oxysporum* (*f. sp. dianthi*, Figure 12A) and *F. oxysporum* (*f. sp. vasinfectum*, Figures 12B and 12C), respectively. The results clearly indicate the effectiveness of 20 µg/mL of NaPdlf against all three fungal pathogens.

EXAMPLE 9

Production of transgenic tobacco plants

(a) *Construction of the binary plasmid*

Primers FLOR1 and FLOR2, as shown below, were used to amplify the full DNA coding sequence of NaPdlf by conventional PCR, in order to incorporate a 5' *EcoRI* and a 3' *XbaI* restriction enzyme site for subsequent cloning steps.

Primer FLOR1 (*EcoRI*-spacer-ATG-seq, 31 mer)

5' GGAATTCTAAACAATGGCTCGCTCCTTGTGC 3' [SEQ ID NO:5]

Primer FLOR2 (*XbaI*-stop-seq, 29 mer)

5' GCTCTAGATCAGTTATCCATTATCTCTTC 3' [SEQ ID NO:6]

The resultant PCR product was directly sub-cloned into a TA vector (pCR2.1-TOPO, Invitrogen) and the sequence was verified by nucleotide sequencing. The *NaPdlf* DNA was excised from the TA vector with *EcoRI* and *XbaI* restriction enzymes, gel purified and subcloned into the pFB98/06 vector (obtained from Florigene Limited, Collingwood, Australia), previously treated with compatible restriction enzymes to create p35-PDF1. This construct (p35-PDF1) contains the defensin cDNA flanked by the 35S cauliflower mosaic virus promoter (CaMV35S) and terminator sequences. Construct p35-PDF1 was

further digested with *Sph*I restriction enzyme, to remove the promoter-gene-terminator cassette, and gel purified. The 3' overhangs produced by *Sph*I were blunt ended with T4 DNA polymerase and gel purified. At this stage, the cassette was inserted into a binary vector, pGCP1988 (Florigene) or pBIN19 (Bevan *et al.* [1983; *supra*]), which had previously been treated with the blunt end cutter *Sma*I restriction enzyme. The resultant constructs were designated (i) pFL1 (contained in pCGP1988), which has a *glean* (*surB*) selectable marker gene under the control of the CaMV35S promoter/terminator, and (ii) pHEX3 (contained in pBIN19) which has a kanamycin (*nptII*) selectable marker gene under the control of the *nos* promoter/terminator. Constructs pFL1 and pHEX3 have the defensin gene cassette in the convergent and tandem orientation relative to their selectable marker genes, respectively. Schematics of constructs pFL1 and pHEX3 are shown in Figure 13.

(b) *Transformation of Agrobacterium tumefaciens* LBA4404

- 15 Electro-competent *Agrobacterium tumefaciens* (LBA4404) were prepared and transformed with either pFL1 or pHEX3 by conventional electroporation in a Gene Pulser (registered trademark)/*E. coli* cuvettes with a 0.2 cm electrode gap (Bio-Rad) and exposed to 1.8 kV, a capacitance of 25 μ FD and a resistance of 600 ohms in a Gene Pulser (Bio-Rad). The
- 20 electroporated cells were combined with 1 mL SOC medium and incubated with shaking at 28°C for 3 h before 150 μ L was withdrawn and plated out on LB agar supplemented with 20 μ g/mL rifampicin for pFL1 transformants or 20 μ g/mL rifampicin and 50 μ g/mL kanamycin for pHEX3 transformants. The plates were incubated overnight at 28°C.
- 25 Transformants were selected by preparing plasmid DNA from randomly chosen colonies, performing diagnostic restriction digests and analysing for inserts by agarose gel electrophoresis.

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(c) *Agrobacterium-mediated transformation of tobacco*

Seeds of *N. tabacum* cultivar Wisconsin 38 (W38) were surface sterilized in 1% v/v sodium hypochlorite for 60 min followed by several washes in sterile water. The sterilized seeds were sown onto MS medium (MS; 0.44% w/v Murashige and Skoog medium (ICN Biomedicals) [*Plant Physiology* 15: 73-97, 1962], 3% w/v sucrose and 0.8% w/v Bacto agar, pH 5.8) and incubated in a temperature control cabinet for 5 weeks. *A. tumefaciens* (LBA4404) transformed with either the pFL1 or pHEX3 constructs were grown for 2-3 days in 10 ml LB medium supplemented with the antibiotics rifampicin (20 µg/ml) or kanamycin (50 µg/ml) and rifampicin at 28°C, respectively. The cells were collected by centrifugation (5 min, 3,500 x g), resuspended in 20 ml MS and incubated with freshly cut leaf disks (1 cm² squares) briefly. The disks were blotted onto sterile 3MM paper before being transferred onto SIM agar (0.44% w/v Murashige and Skoog medium (ICN Biomedicals) [1962; *supra*], 3% w/v sucrose, 1.0 mg/L BAP, 0.5 mg/L indole acetic acid (IAA) and 0.8% w/v Bacto agar, pH 5.8) and incubated for 3 days at 25°C in light. Control leaf disks were treated similarly with MS alone. Following co-cultivation, calli were transferred onto selective media to induce shoot formation. pFL1 transformed calli were transferred to SIM agar supplemented with 1.5 mg/L glean and 250 mg/L cefotaxamine while the pHEX3 transformed calli were selected with 100 mg/L kanamycin and 250 mg/L cefotaxamine. The regenerated shoots were dissected from the calli, briefly dipped in IBA (1 mg/mL) solution, and transferred onto root-inducing medium (RIM; 0.44% w/v Murashige and Skoog medium [1962; *supra*], 3% w/v sucrose, 150 mg/L timentin and 0.8% w/v Bacto agar), supplemented with either 1.5 mg/L glean or 100 mg/L kanamycin for pFL1 or pHEX3, respectively and incubated as described previously. When adequate root growth was established, plantlets were potted into soil and grown under standard glasshouse conditions.

(d) *Detection of NaPdf1 in transgenic tobacco leaves*

Leaves (4th or 5th position) were cut at the petiole from glasshouse grown plants. The tissue was frozen in liquid nitrogen and ground to a fine powder with a mortar and pestle. The

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tissue was added to extraction buffer: 50 mM Tris-HCl pH 8.0, 10 mM EDTA and 0.5 M NaCl (1 mL/g of fresh wet weight). The mixture was allowed to stand on ice for 15 min before clarification by centrifugation at 13,500 x g for 15 min, 4°C. Protein concentrations were estimated by the method of Bradford (1976), reagents were from Bio-Rad and BSA was used as a standard. Total soluble leaf proteins (100 µg) were separated by SDS-PAGE using either 15% w/v or preformed 4-12% w/v polyacrylamide gradient gels (Novex) as described in Example 4(d), above. Following electrophoresis, proteins were either stained using coomassie blue or transferred to nitrocellulose for immunodetection of NaPdf1 as described in Example 4(e), above. Comparative results are shown in Figures 14A and B.

EXAMPLE 10

Insect feeding trials

(a) *Source of insects*

Helicoverpa punctigera larvae came from a colony collected in Victoria, Australia and maintained in culture at La Trobe University, Melbourne and *H. armigera* were obtained from a colonies maintained at La Trobe University, Melbourne or the Australian Cotton Research Institute, at the Entomology Department of the Commonwealth Scientific and Industrial Research Organisation in Narrabri, NSW.

(b) *Bioassays with transgenic tobacco leaves*

Three experiments were conducted with clonal material from transgenic plant, pFL1/W19 (transformed with the pFL1 construct) and transgenic plant, pHEX3.4 (transformed with the pHEX3 construct). An untransformed W38 plant was used as negative control.

In experiments 1 and 2, 31 and 40 newly hatched *Helicoverpa punctigera* larvae were selected for each treatment, respectively. The larvae were reared in individual plastic cups with lids (Solo (registered trademark) plastic portion cups, 28 mL) containing 1.5% w/v Bacto agar and were fed leaf segments that were replaced either every 2-3 days or when

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more than 75% had been consumed. The amount of leaf material was increased as the larvae reached 5th instar. Young leaves from non-flowering plants were used in all bioassays. To avoid a wounding response, the leaves were freshly excised from the petiole with a clean scalpel blade and were divided into sections (2 x 2 cm) by careful dissection between the major veins to minimize any wound response in the leaf sections. The larvae were kept in a controlled temperature room of $24 \pm 1^\circ\text{C}$, under light. The weights of the larvae were measured every 2-3 days until day 23 and the mean weight calculated. In experiment 3, 40 *H. armigera* larvae were used under the same conditions described for the *H. punctigera* bioassays. Results are shown in Figure 15.

(c) *Bioassays with artificial diet*

To confirm that the insecticidal activity was due to the defensin and not to upregulation of other endogenous defence molecules, a feeding trial using artificial cotton leaf diet containing purified defensin from floral buds was performed. The 6 kDa cysteine rich proteinase inhibitors (NaPI) from *N. alata* (Atkinson *et al.*, *Plant Cell* 5: 203-213, 1993) were used as positive control and casein was used as a negative control. Feeding trials were conducted as described in Heath *et al.* (*J. Insect Physiology* 43: 833-842, 1997) except that the artificial diet was based on cotton leaves (Table 2). Thirty milligram of defensin was purified from floral buds of *N. alata* and used in artificial diets at two concentrations. *H. armigera* larvae fed on defensin at 0.3% (w/v) were 40% smaller than controls (Figure 16) while larvae fed on NaPI at the same concentration were about 60% smaller than controls.

EXAMPLE 11

Production of transgenic cotton plants

(a) *Agrobacterium-mediated transformation of cotton*

Seeds of *Gossypium hirsutum* cultivar Coker 315 were surface sterilized in 2% v/v sodium hypochlorite for 60 min followed by several washes in sterile water. The sterilized seed

- were sown onto half-strength MS medium (MS; 0.22% w/v Murashige and Skoog salt mixture (Gibco BRL) [1962; *supra*], 0.2% Gelrite (Phyto Technology Laboratories), pH 5.8) and incubated at 30°C in the dark for 7 days. *A. tumefaciens* (LBA4404) transformed with the pHEX3 construct was grown overnight in 25 ml LB medium supplemented with the antibiotic kanamycin (50 µg/mL) at 28°C. The absorbance at 550 nm was measured and the cells were diluted to 2×10^8 cells per ml in MS liquid media (0.43% w/v Murashige and Skoog salts [1962; *supra*], pH 5.8). Cotton hypocotyls were cut into 1.5-2 cm pieces and mixed briefly (0.5-3 min) in the diluted *Agrobacterium* culture. The explants were blotted dry on sterile 3MM paper and transferred to medium 1 (0.43% w/v Murashige and Skoog salt mixture (GibcoBRL) [1962; *supra*], 0.1% v/v Gamborg's B5 vitamin solution (Sigma), 0.1 g/L myo-inositol, 0.9 g/L MgCl₂, (hexahydrate), 1.9 g/L potassium nitrate, 0.2% w/v Gelrite, 3% w/v glucose, pH 5.8) overlaid with sterile filter paper and incubated for 3 days at 26°C under lights.
- Following co-cultivation, explants were transferred to medium 2 (medium 1 plus 0.1 mg/L kinetin, 0.1 mg/L 2,4-D, 500 mg/L carbenicillin, 35 mg/L kanamycin) and maintained at 30°C under low light. After 4 weeks explants were transferred to medium 3 (medium 1 plus 500 mg/L carbenicillin, 35 mg/L kanamycin) and maintained at 30°C under low light. Explants and callus were sub-cultured every 4 weeks on medium 3 and maintained at 30°C under low light. Embryos were excised from the tissue and germinated in medium 4 (1.2 mM CaCl₂·2H₂O, 5.0 mM KNO₃, 2.0 mM MgSO₄·7H₂O, 3.0 mM NH₄NO₃, 0.2 mM KH₂PO₄, 4 µM nicotinic acid, 4 µM pyridoxine HCl, 4 µM thiamine HCl, 30 µM H₃BO₃, 30 µM MnSO₄·H₂O, 9 µM ZnSO₄·7H₂O, 1.5 µM KI, 0.9 µM Na₂MoO₄·2H₂O, 0.03 µM CuSO₄·5H₂O, 0.03 µM CoCl₂·6H₂O, 0.5% w/v glucose, 0.3% w/v Gelrite, pH 5.5) and maintained at 30°C under high light.

Germinated embryos were then transferred to Magenta boxes containing medium 5 (1.2 mM CaCl₂·2H₂O, 40.0 mM KNO₃, 2.0 mM MgSO₄·7H₂O, 15 mM NH₄Cl, 0.2 mM KH₂PO₄, 4 µM nicotinic acid, 4 µM pyridoxine HCl, 4 µM thiamine HCl, 30 µM H₃BO₃, 30 µM MnSO₄·H₂O, 9 µM ZnSO₄·7H₂O, 1.5 µM KI, 0.9 µM Na₂MoO₄·2H₂O, 0.03 µM CuSO₄·5H₂O, 0.03 µM CoCl₂·6H₂O, 2.0% w/v sucrose, 0.2% w/v Gelrite, pH 5.5) and

maintained at 30°C under high light. Once a plant has formed a good root system and produced several new leaves it was transferred to soil in pots and acclimatised in a growth cabinet at 28°C and then grown in a glasshouse at (27-29°C day, 20-24°C night).

5 (b) *Detection of NaPdf1 in transgenic cotton*

Leaves (first position, 3-4 cm in diameter) were excised from plants grown either in the growth cabinet or in the glasshouse. The tissue (100 mg) was frozen in liquid nitrogen and ground to a fine powder with a mortar and pestle. The powder was added to 2 x sample
10 buffer (300 µl, Novex NuPAGE LDS sample buffer, 10% v/v β-mercaptoethanol), vortexed for 30 sec, boiled for 5 min and then centrifuged at 14,000 rpm for 10 min and the supernatant retained. Total soluble leaf extracts were separated by SDS-PAGE on preformed 4-12% w/v polyacrylamide gradient gels (Novex, NuPAGE bis-tris, MES buffer) for 35 min at 200V in a Novex X Cell II mini-cell electrophoresis apparatus.
15 Prestained molecular weight markers (Novex SeeBlue) were included as a standard. Proteins were transferred to nitrocellulose membrane (Micron Separations Inc. 0.22 micron pore size) for 60 min at 30V using the Novex X Cell II mini-cell electrophoresis apparatus in NuPAGE transfer buffer with 10% v/v methanol. After transfer, membranes were incubated for 1 min in isopropanol, followed by a 5 min wash in TBS.

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The membrane was blocked for 1 h in 3% w/v BSA at RT followed by incubation with primary antibody overnight at RT (1:2500 dilution in TBS). The membrane was washed 5 x 10 min in TBST before incubation with goat anti-rabbit IgG conjugated to horseradish peroxidase for 60 min at RT (Pierce, 1:100,000 dilution in TBS). Five further 10 min
25 TBST washes were performed before the membrane was incubated with the SuperSignal West Pico Chemiluminescent substrate (Pierce) according to the Manufacturer's instructions. Membranes were exposed to ECL Hyperfilm (Amersham Pharmacia Biotech). Results are shown in Figure 14C.

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EXAMPLE 12

Insect feeding trial with transgenic cotton

One experiment was conducted with two independently transformed T2 generation transgenic cotton lines. The plants were produced by selfing the primary transgenic lines CT35.9.4 and CT35.125.1 (both transformed with the pHEX3 construct) and consisted of a mixture of homozygous and hemizygous plants. Parent untransformed Coker 315 plants were used as a negative control.

Twenty newly hatched *H. armigera* larvae were selected. The larvae were reared in individual plastic cups with lids (Solo (registered trademark) plastic portion cups, 28 ml) containing 1.5% Bacto agar and were fed leaf segments that were replaced either every 2-3 days or when more than 75% had been consumed. Young leaves from non-flowering plants were used in the bioassay. The larvae were kept in a controlled temperature room at $25 \pm 1^\circ\text{C}$, under light. The number of dead larvae was recorded on days 4, 6 and 8. The weight of each surviving larvae was measured at day 8 and the mean weight calculated. Results are shown in Figure 17.

EXAMPLE 13

Production of insect-resistant plants

(a) Molecular cloning of genes from a range of species

Amino acid sequences of any one of the sequences set forth in Figure 10 (SEQ ID NO:25 to SEQ ID NO:49) are used to design suitable oligonucleotide primers for use in screening cDNA or genomic DNA libraries, as appropriate. Using, for example PCR, corresponding full nucleotide coding sequences are cloned as, for example, described in Examples 4(a) and 9(a), above. Expression cassettes are then inserted into desired vectors such as, for example, pBIN19 or pCGP1988, use of which is described in Example 9(a).

(b) *Transformation and regeneration of insect-resistant plants*

Using standard techniques known and available to those skilled in the art, selected plant material of target plant species is transformed with one or more vectors comprising the expression cassettes carrying the anti-insect sequences from the corresponding species. Suitable transformation methods include, but are not limited to, the *Agrobacterium*-mediated transformation protocol set forth in Example 9(b), 9(c) and 9(d), modified as necessary for particular plant species. Other means of transformation of particular plant species are well known and include, for example, biolistic transformation procedures.

Following regeneration, plants are assayed for resistance to attack by common plant pests including insects.

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

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